Universidade do Minho
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Elena Geta Popa

Engineering articular cartilage using newly developed carrageenan basedhydrogels

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LOGO

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To my dearest, Adrian

Nihil Sine Deo

The motto of the Royal family

of Romania

ACKNOWLEDGEMENTS

All most six years have passed quickly since I started my PhD studies in the University of Minho (3Bs Research Group, Portugal). I still remember when I first came here, since it was in November and the wheatear was great. I was fascinated by the fact of living abroad, getting to know new people, new places and new culture. Now, time almost comes to the end of my PhD period. This thesis is completed with prayers of many and sacrificial love of my family and friends, as well as with the help and support by my supervisors and colleagues. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them.

First of all, I like to express my special gratitude to my supervisor, Professor Rui Reis for the opportunity of completing my PhD in 3B's research group and I am truly grateful for such life changing experience. Your endurance, view on research and continuous work is impressive and has made a deep impression on me and led me to such interesting field of research full of challenges. I will always remember your words «Gosto de competir, lutar e ganhar» which I believe are summarizing your life/research philosophy and are beyond doubt inspiring. Thank you for believing in my abilities and for the chance to overcome many scientific struggles. This was a unique and enriching test both personally and professionally, for all the challenge conquered. Moreover, I appreciate the many opportunities to present and discuss this work at national and international symposia.

I am heartily thankful to Manuela, whose encouragement, guidance and support from the initial to the final level enabled me to develop systematically. I deeply appreciate your guidance in the scientific matters and stimulating discussions, which allows me to see different aspects of the research project in the broader perspective. I am deeply touched by your dedication, strength and determination especially under pressure, time limit and when I most needed to move forward. I'm very grateful not only for your constant help and patient guidance during my PhD study, but also for your friendliness and concerns about the things not related to research. I have to recognize that I could not have done this thesis/work without you.

I gratefully acknowledge that this work was performed at the 3Bs Research Group, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Portugal, and was funded by the Portuguese Foundation for Science and Technology (FCT) and Marie Curie EST join(ed)t Project for awarding me a PhD grant.

I am very grateful to my collaborators on the research papers described in this thesis: Prof. João Mano, Alexandra Marques, Tírcia Santos, Sofia Caridade, Mariana Oliveira who have been instrumental in providing indispensable support for my research, Elsa Ribeiro for her kind helps in the SEM analysis.

I would like to express my most sincere gratitude to a special group of people, good friends like Pedro Carvalho, Simone Silva, Daniela Coutinho, Ana Dias, Vitor Espírito Santo I really like the time shared with you. I learned a lot from you all, Pedro, to be there when its needed, Simone, be happy and carry on, Daniela, have always a smile no matter what, Ana, be dedicated and organized, Vitor, singing in the lab makes cell grow faster. Also I could never forget Márcia and I want to express particular thanks for all the support and company during coffee breaks, you are a dedicated, enthusiastic and true friend which anyone would really want, thank you for your patience.

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I offer my regards to the management team, administration staff and to the informatic support for invaluable help and technical assistance. I also like to make an unusual thanks to my good friend *caffeine*.

I thank to all of those who contributed in any respect during the completion of the project.
MUITO OBRIGADA A TODOS!

I deeply thank to my beloved husband, Adrian, for his unconditional love, endless support, understanding and sacrifice throughout the work with this thesis. Last, but not least, I am thankful to my family (including my cat), my parents, my sister for all the patience, encouragement and comprehension, for listen and forgiving my flaws.

ABSTRACT

Articular cartilage holds specific functionality in the human body creating smooth gliding areas and allowing the joints to move easily without pain. However, due to its avascular nature and to the low metabolic activity of the constituent cells-the chondrocytes, cartilage has a low regenerative potential. The current surgical options to treat damaged cartilage are not long lasting and involve frequent revisions. Tissue engineering may provide an alternative approach for cartilage repair, involving the use of hydrogels which can act as a temporary artificial matrix for encapsulated cells, supporting their growth and inducing the extracellular matrix (ECM) production. The use of hydrogels is regarded as advantageous for cartilage tissue engineering because they provide similar environment to the native ECM where cells reside. Up to date, many different hydrogel systems based on natural polymers have been developed for cartilage regeneration. Among these systems, marine polysaccharides origin hydrogels have received increasing attention since they are seen as an inexpensive potential source of material with owing to their specific characteristics and composition.

In the present PhD thesis it was investigated the potential of carrageenan, a sulphated polysaccharide that can be extracted from red algae, as an alternative hydrogel for cartilage regeneration. Carrageenans offer appealing assets for cell encapsulation/delivery, derived from their chemical composition and gelation process.

Therefore, the main aims of this thesis were:

- develop and characterize different blends of carrageenan with alginate, processed into different formats;
- study the chondrogenic differentiation of stem cells loaded in κ-carrageenan hydrogels;
- asses the viability, proliferation and chondrogenic potential of different cell types, laden in κ-carrageenan hydrogels;
- evaluate the effect of cryopreservation methods on the viability and chondrogenic differentiation of stem cells encapsulated in κ-carrageenan hydrogels;
- study the *in vitro* and *in vivo* biocompatibility of κ-carrageenan hydrogels.

In the first part of this thesis, different formulations of alginate and carrageenan hydrogels and different processing parameters were considered in order to determine the best conditions required to achieve the most adequate response in terms of the mechanical stability, cell viability and functionality of the

developed systems. The morphology, size and structure of the hydrogels and their degradation behavior and mechanical properties were evaluated as well as their cytotoxicity and ability to encapsulate chondrocytes. The results obtained indicated that the different formulations, both in the form of beads and fibers have considerable potential as cell-carrier materials for cell delivery in tissue engineering/regenerative medicine applications. The results obtained in this study also showed that κappa carrageenan is more suitable for the proposed application than ιota carrageenan.

Subsequently, further studies were designed to assess the ability of κappa-carrageenan hydrogels to support the chondrogenic differentiation of human adipose derived stem cells (hASCs). Moreover, the chondrogenic potential of hASCs encapsulated in κ-carrageenan hydrogels was compared to hydrogels laden with other cell types, namely human primary (nasal) chondrocytes (hNCs) and a chondrocytic cell line (ATDC5). The *in vitro* cellular behavior of the encapsulated cells within κ-carrageenan hydrogel was analyzed after different culturing periods by mechanical tests and using biochemical assays as well as cytohistological and real time RT-PCR analysis.

The results from the analysis of the cells encapsulated in the developed systems indicated that κ-carrageenan hydrogels support the viability, proliferation and chondrogenic differentiation of hASCs. Interestingly, the mechanical analysis demonstrated an increase in stiffness and in the viscoelastic properties of κ-carrageenan gels with encapsulated hASCs along the time in culture with chondrogenic media, as compared to hydrogels without cells or cell laden hydrogels cultured in basal media.

Furthermore, these studies have also demonstrated that the 3 types of cells encapsulated in κ-carrageenan hydrogels showed good cellular viability and proliferation up to 21 days of culture and the cell laden hydrogels showed to be positive for specific cartilage markers. Nevertheless, the results also showed that hASCs embedded in κ-carrageenan hydrogels proliferate faster and exhibit higher expression levels of the typical cartilage markers analysed as compared to hNCs or ATDC5 cells. Based on this data, it was possible to conclude that κ-carrageenan hydrogel provides a good support for culture and differentiation of encapsulated cells and that hASCs may provide an advantageous alternative to primary chondrocytes, currently used in clinical treatments of cartilage defects/diseases.

These bioengineered constructs are anticipated to participate in a cartilage regeneration strategy providing temporary habitation for cell survival, proliferation and production of extracellular matrix which is expected to replace the hydrogel, enhancing the regeneration of native tissues in clinical settings. Nevertheless, the time span needed for obtaining a functional cartilage substitute using tissue engineering strategies, together with the need for specific patient oriented constructs stimulated our interest for assessing the possibility of developing of "off-the shelf" products, based on cryopreservation, that would provide clinical substitute available as needed and could be adapted to an

autologous immediate solution for the patient. Therefore, the following experiments were planned to examine the effects of cryopreservation on the chondrogenic differentiation characteristics of hASCs encapsulated in κ-carrageenan hydrogels. The results obtained show that the hydrogels withstand the cryopreservation with dimethyl sulfoxide, maintaining their structural integrity, while assisting cells proliferation and chondrogenic potential after cryopreservation. Thus, cell encapsulation systems of natural based hydrogels seem to be an interesting approach for the preservation of cartilage tissue engineered products.

The final stage of the work developed involved studies on the *in vitro* and *in vivo* biocompatibility of κ-carrageenan hydrogels. The *in vitro* cytotoxicity of the hydrogels was evaluated under standard tests using the L929 cell line, and chemiluminescence assays were performed using human polymorphonuclear cells. The *in vivo* study was accomplished by the subcutaneously implantation of carrageenan hydrogels discs in Wistar rats for up to 15 days. The obtained findings indicated that κ-carrageenan hydrogels induced a reduced and insignificant signal concerning the detection of superoxide and hydroxyl anions and seems to induce a low inflammatory response and thus, can be further studied to be used in target biomedical applications.

Moreover, a recent preliminary *in vivo* study showed that loading κ-carrageenan with cells previously exposed to chondrogenic medium led to higher stability of the construct *in vivo* (rat subcutaneous model) as compared to hydrogels cultured in basal media or hydrogels without cells. Such accomplishment is probably related to an increase in the extracellular matrix deposition which may result in progressive increase of the mechanical properties, in agreement with the results obtained in the *in vitro* studies performed with κ-carrageenan.

Overall, the work developed under this thesis allowed to conclude that κ-carrageenan hydrogels laden with human adipose derived stem cells show a great potential to be tailored to specific applications in cartilage tissue regeneration approaches.

RESUMO

A cartilagem articular desempenha funções específicas no corpo humano criando áreas de deslizamento lisas e quase sem atrito que permitem a movimentação das articulações sem causar dor. No entanto, devido à sua natureza avascular e a reduzida atividade metabólica das células que a constituem condrócitos, possui um potencial de regeneração baixo. As atuais opções cirúrgicas para o tratamento da cartilagem danificada não são duradouras e geralmente obrigam a repetição de procedimentos e/ou recorrência dos sintomas. A engenharia de tecidos proporciona uma abordagem alternativa na regeneração da cartilagem, envolvendo o uso de hidrogéis que atuam como uma matriz artificial temporária para encapsular células, suportar a sua proliferação e produção de matriz extracelular. O uso de hidrogéis é considerado vantajoso na engenharia de tecido cartilagíneo, uma vez que estes proporcionam um ambiente similar ao da matriz extracelular onde as células nativas residem. Até à data, foram desenvolvidos vários hidrogéis com base em polímeros naturais para regenerar cartilagem. Entre estes, os polissacáridos de origem marinha têm recebido atenção crescente já que são considerados uma fonte barata e praticamente inesgotável de material, e também pelas suas características e composição.

Nesta tese de doutoramento foi investigado o potencial da carragenina, um polissacárido sulfatado extraído das algas vermelhas, como hidrogel alternativo para regeneração de cartilagem. As carrageninas possuem aributos apelativos para o encapsulamento/libertação de células, dada a sua composição química e processo de gelificação.

Assim, os principais objetivos desta tese foram:

- desenvolver e caracterizar diferentes misturas de carragenina com alginato, processadas de diferentes formas;
- estudar a diferenciação condrogénica de células estaminais encapsuladas/cultivadas em hidrogéis de κ-carragenina;
- avaliar a viabilidade, proliferação e potencial condrogénico de diferentes tipos celulares encpasuladas em hidrogéis de κ-carragenina;
- analisar o efeito de métodos de criopreservação na viabilidade e capacidade de diferenciação condrogénica de células estaminais encapsuladas em hidrogéis de κ-carragenina;
- investigar a biocompatibilidade in vitro e in vivo de hidrogéis de κ-carragenina.

Na primeira parte desta tese, foram consideradas diferentes formulações de hidrogéis de alginato e carragenina, bem como diferentes parâmetros de processamento de forma a determinar as condições ótimas para obter a resposta mais adequada em termos de estabilidade mecânica, viabilidade celular e funcionalidade dos sistemas desenvolvidos.

A morfologia, tamanho e estrutura dos hidrogéis, a sua degradação e propriedades mecânicas foram avaliadas, bem como a sua citotoxicidade e capacidade de mantera viabilidade de durante o encapsulamento de condrócitos. Os resultados obtidos indicaram que diferentes formulações, quer sob a forma de esferas quer sob a forma de fibras, possuem um potencial considerável como sistemas de encapsulamento/libertação de células em aplicações de engenharia de tecidos/medicina regenerativa. Os resultados obtidos revelaram que a κapa-carragenina é mais adequada para a aplicação proposta do que a ιota-carragenina.

Consequentemente, os estudos posteriores foram planeados para avaliar a capacidade dos hidrogéis de κ-carragenina de suportar a diferenciação condrogénica de células estaminais humanas derivadas de tecido adiposo (hASCs). Para além disso, o potencial condrogénico de hASCs encapsuladas em κ-carragenina foi comparado com o de hidrogéis semeados com outros tipos celulares, nomeadamente condrócitos (nasais) primários humanos (hNCs) e uma linha celular condrocítica (ATDC5). O comportamento celular in vitro das células encapsuladas foi investigado após diferentes períodos de cultura, através de testes mecânicos, ensaios bioquímicos, citohistologia e RT-PCR.

Os resultados destes ensaios indicaram que os hidrogéis de κ-carragenina mantêm a viabilidade, proliferação e diferenciação condrogénica das hASCs. Interessantemente, a análise mecânica mostrou um aumento das propriedades mecânicas dos géis de κ-carragenina contendo hASCs encapsuladas, ao longo do tempo de cultura com meio condrogénico, quando comparados com os géis sem células ou géis com células mas em meio basal.

Os resultados demonstraram igualmente que os três tipos celulares encapsulados em hidrogéis de κ-carragenina mantiveram boa viabilidade celular e proliferação até aos 21 dias de cultura, e os géis semeados com as células expressaram positivamente marcadores específicos de cartilagem.

No entanto, os resultados mostraram que as hASCs encapsuladas na κ-carragenina proliferaram mais rapidamente e exibiram níveis de expressão de marcadores típicos de cartilagem mais elevadoss do que os observados em hNCs e ATDC5. Com base nestes dados foi possível concluir que o hidrogel de κ-carragenina providencia um bom suporte para a cultura e diferenciação de células nele encapsuladas, e que as hASCs podem ser uma alternativa vantajosa aos condrócitos primários usados atualmente em tratamentos clínicos de defeitos/patologias da cartilagem.

Antecipa-se que estes hidrogéis possam ser utilizados em estratégias de regeneração de cartilagem, providenciando habitat temporário para as células encapsuladas, aseegurando a sua viabilidade, proliferação e produção de matriz extracelular, que deverá substituir o hidrogel, intensificando a regeneração do tecido nativo em situações clínicas. Apesar das vantagens, é necessario um certo tempo para obter um substituto funcional da cartilagem com este tipo de abordagens de engenharia de tecidos, há uma clara necessidadede produtos "off-the-shelf", que possam ser imediatamente disponibilizados e adaptados a uma solução autóloga para o paciente. Isto pode ser evantualmente conseguido recorrendo a métodos baseados na criopreservação. Por este motivo, as experiências seguintes foram planeadas para examinar os efeitos da criopreservação na diferenciação condrogénica característica das hASCs encapsuladas em κ-carragenina. Os resultados obtidos mostraram que o hidrogel suporta a criopreservação com dimetilsulfóxido (DMSO) mantendo a sua integridade estrutural e a capacidade de manter a proliferação e diferenciações celulares. O último estádio do trabalho desenvolvido envolveu estudos in vitro e in vivo de biocompatibilidade dos hidrogéis de κ-carragenina. A citotoxicidade dos hidrogéis foi avaliada por ensaios standards utilizando uma linha celular (L929) e por ensaios de quimoluminescência com células polimorfonucleadas humanas. O estudo in vivo envolveu a implantação subcutânea de discos de κ-carragenina em ratos Wistar durante 15 dias. Os resultados obtidos demosntraram que os hidrogéis de κ-carragenina induziram uma resposta insignificante na detecção de aniões superóxido e hidroxil, bem como resposta inflamatória mínima podendo ser estudados para aplicações biomédicas.

Um estudo preliminar recente, in vivo revelou que a κ-carragenina semeada com células pré-diferenciadas em meio condrogénico apresenta maior estabilidade in vivo (modelo de implante subcutâneo em rato), comparando com géis previamente cultivados com meio basal ou géis sem células. Este facto deve-se muito provavelmente ao aumento de deposição de matriz extracelular que pode resultar no aumento das propriedades mecânicas, em concordância com os resultados obtidos in vitro.

Resumindo, o trabalho desenvolvido e apresentado nesta tese, permitiu concluir que os hidrogéis de κ-carragenina encapsulados com células humanas derivadas do tecido adiposo, revelam grande potencial para serem adaptados a aplicações específicas de terapias de regeneração de cartilagem.

TABLE OF CONTENTS

#

2D - bidimensional

3D - tridimensional

Ø – diameter

21G - 21 gauge

-ΔΔCt - delta delta critical threshold

A

α-MEM - alpha-Modification Eagle Medium

A/B - antibiotic/antimicotic

ACI - autologous chondrocyte implantation

AGCR - agreccan

ASC - adipose stem cells

B

bFGF - basic fibroblast growth factor

BMSCs – bone marrow derived mesenchymal stem cells

BMI - body mass index

BSA - bovine serum albumin

C

Ca^{2+} - calcium ions

$CaCl_2$ - calcium chloride

Calcein AM - Calcein acetoxymethyl ester

CaReS® - Cartilage Repair System

cDNA - complementary deoxyribonucleic acid

Cl^- - chloride ion

Col I - collagen type I

Col II - collagen type II

CO_2 - carbon dioxide

CM – chondrogenic medium

cm - centimeter

cm^2 - square centimeter

Ct - critical threshold

D

DAB - 3,3'-diaminobenzidine

DAPI - 4',6-diamidino-2-phenylindole

Dex – Dexamethasone

DMA - dynamic mechanical analysis

DMB - 1,9-dimethylmethylene blue

DMEM - Dulbecco's Modified Eagle Medium

DMSO - dimethyl sulfoxide

DNA - deoxyribonucleic acid

dsDNA - double-stranded DNA

E

E' - storage modulus

E'' - loss modulus

ECACC - European Collection of Cell Cultures

ECM - extracellular matrix

EDTA - ethylenediaminetetraacetic acid

Ex/Em - excitation/emission

F

FBS - foetal bovine serum

fMLP - formyl-methionyl-leucyl-phenylalanine

G

g - gram

GAGs - glycosaminoglycans

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GF - growth factor

x g - centrifugal force

H

h - hour

hASCs - human adipose derived stem cells

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H&E - hematoxylin-eosin

hMSCs - human mesenchymal stem cells

hNC - human nasal chondrocytes

hTGF-β1 - human transforming growth factor beta-1

Hz - hertz

I

IFN-γ - interferon gamma

IL-1a - interleukin-1 alpha

IL-4 - interleukin-4

ITS - insulin, transferrin, and selenium solution

K

kPa - kilo pascal

K^+ - potassium ion

KCl - potassium chloride

L

L929 - rat lung fibroblasts cell line

M

MACI - matrix-assisted autologous chondrocyte implantation

Mg^{2+} - magnesium

MSCs - mesenchymal stromal cells

mRNA - messenger ribonucleic acid

µg - microgram

µm - micrometer

mL - milliliter

mg - miligram

mm - milimiter

mM - milimolar

MEM - minimum essential medium

MTS - (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

N

nm - nanometer

$NaHCO_3$ - sodium bicarbonate

O

OD - optical density

P

P - passage

Pa - pascal

PBS - phosphate buffer saline

PCR - polymerase chain reaction

PMA - phorbol 12-myristate 13-acetate PMNs - polymorphonuclear neutrophils

R

RNA - ribonucleic acid

rpm - revolutions per minute

ROS - reactive oxygen species

RT-PCR - reverse transcriptase polymerase chain reaction

RNA - ribonucleic acid

S

SEM - scanning electronic microscopy

SOX9 - SRY (sex determining region Y)-box 9

SO_4^{2-} - sulphate

s - second

T

TE - tissue engineering

TCP - tissue culture polystyrene

TGF-β1 - transforming growth factor beta-1

V

v/v - volume per volume

W

wt - weight

wt/v - weight per volume

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

CHAPTER VI

CHAPTER VII

CHAPTER VIII

LIST OF TABLES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER VI

CHAPTER VII

SHORT *CURRICULUM VITAE*

Elena Geta Popa was born in 1980 Sibiu, Romania and currently is a PhD student in 3B's Research Group – Biomaterials, Biodegradables and Biomimetics at University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Guimarães, Portugal.

In 2010 she was awarded with a PhD grant from the Portuguese Science Foundation (FCT) in Tissue Engineering, Regenerative Medicine and Stem Cells. In 2008 was the beneficiary of a Marie Curie PhD scholarship under the Marie Curie EST join(ed)t Project and started the PhD at 3B's Research Group with main focus on cartilage tissue engineering under the supervision of Prof. Rui Reis (Director of 3B's Research Group and Full Professor at University of Minho) and Prof. Manuela Gomes (Invited Assistant Professor, Portugal-MIT Program). During the course of PhD, she participated in several training actions namely, in July 2010 at Termis-EU EXPERTISSUES Summer School MAKING AN IMPACT: The Preparation of High Quality Papers in Tissue Engineering & Regenerative Medicine, Halifax Hall, The University of Sheffield, Sheffield UK, in September 2009, in the 3rd Marie Curie Cutting Edge Practical Training Course on Fabrication and Characterization of tissue engineering scaffolds, 3B's Research Group – AvePark, Caldas das Taipas, Guimarães, Portugal and in November 2008, at JOIN(ed)T Training Workshop in RT-PCR, FACS, Western blotting, and Laser-Scanning Confocal Microscopy and oral presentations by the JOIN(ed)T fellows, Johannes Gutenberg Univeristy (JGU),Mainz, Germany. She received the following awards: abstract selected as one of the 50 Best Abstract Award submitted to the TERM International Society – EU Meeting, 2010 Galway, Ireland; STAR Award & Honorable Mention Presentation at Society For Biomaterials 2012 Fall Symposium, New Orleans, USA. Furthermore, she was involved in the organization of several international scientific meetings as the TERMIS 2008 and ESAO-IFAO 2011 in Porto.

In 2007 she received the Master degree in Natural products with the dissertation project entitled "The reactive extraction of cinnamic acid" at Faculty of Industrial Chemistry, Gh. Asachi Technical University, Iasi, Romania. In 2006 she obtained the BSc degree as a Medical bioengineer at Faculty of Medical Bioengineering, Biomedical Instrumentation Department, Gr. T. Popa University of Medicine and Pharmacy, Iasi, Romania, with the thesis "Biocompatible magnetic nanocarriers and their applications in medicine".

The research work undertaken resulted in 6 publications in peer-reviewed international journals and she has participated in the most relevant international conferences in the Biomaterials and Regenerative Medicine field, with a total of 10 communications (oral and poster presentations).

LIST OF PUBLICATIONS

This thesis is based on the following original publications:

INTERNATIONAL REFEREED JOURNALS

1. **Popa E. G.**, Gomes M. E., and Reis R. L., "Cell Delivery Systems Using Alginate–Carrageenan Hydrogel Beads and Fibers for Regenerative Medicine Applications", Biomacromolecules, vol. 12, issue 11, pp. 3952-3961, 2011.

2. **Popa E. G.,** Caridade S. G., Mano J. F., Reis R. L., and Gomes M. E., "Chondrogenic potential of injectable κ-carrageenan hydrogel with encapsulated adipose stem cells for cartilage tissue-engineering applications", Journal of Tissue Engineering and Regenerative Medicine, doi:10.1002/term.1683, 2013.

3. **Popa E. G.**, Reis R. L., and Gomes M. E., "Chondrogenic phenotype of different cells encapsulated in κ-carrageenan hydrogels for cartilage regeneration strategies", Biotechnology and Applied Biochemistry, vol. 59, issue 2, pp. 132-141, 2012.

4. **Popa E. G.,** Rodrigues M. T., Coutinho D. F., Oliveira M. B., Mano J. F., Reis R. L., and Gomes M. E., "Cryopreservation of cell laden natural origin hydrogels for cartilage regeneration strategies", Soft Matter, doi:10.1039/c2sm26846a, 2012.

5. **Popa E.G.**, Carvalho P.P., Dias A.F., Santos T.C., Santo V.E., Marques A.P., Dias IR, Viegas C A A, Gomes M.E. and Reis R.L., "Evaluation of the *in vitro* and *in vivo* biocompatibility of carrageenan based hydrogels". *Submitted, 2013.*

6. **Popa E.G.**, Reis R.L. and Gomes M.E., "Polysaccharide-based hydrogels used for the regeneration of articular cartilage". *Submitted, 2013.*

In addition, some unpublished data are presented in this thesis.

COMMUNICATIONS IN INTERNATIONAL CONFERENCES

Oral Presentations

1. **Popa EG**, Carvalho P.P., Dias AF, Santo VE, Frias AM, Marques AP, Dias IR, Viegas C A A, Gomes ME and Reis RL, *In vitro* and *in vivo* biocompatibility evaluation of κ-carrageenan hydrogels aimed at applications in regenerative medicine, TERMIS-EU Annual Meeting, Granada, Spain, June 2011.

2. **Popa EG**, Reis RL and Gomes ME, Novel hydrogels based on carrageenan with encapsulated adipose derived stem cells for cartilage tissue engineering, 2nd scientific meeting of the Institute for Biotechnology and Bioengineering, Braga, Portugal, October 2010.

3. **Popa EG**, Caridade SG, Mano JF, Gomes ME and Reis RL, Novel hydrogels based on carrageenan with encapsulated adipose derived stem cells (ASCs): evaluation of chondrogenic potential and mechanical properties, Termis-EU 2010, Galway, Ireland, June 2010.

Poster Presentation

1. **Popa EG**, Rodrigues MT, Coutinho DF, Neves NM, Gomes ME and Reis RL, Cryopreservation of cell laden natural origin hydrogels for cartilage regeneration strategies, Society for Biomaterials, New Orleans, Louisiana, USA, October 2012.

2. **Popa EG**, Reis RL and Gomes ME, *In vitro* performance of κ-Carrageenan hydrogels combined with different types of cells aimed at application in cartilage regeneration, 4th Joint ESAO-IFAO Congress 2011, Porto, Portugal, October 2011.

3. **Popa EG**, Rodrigues MT, Gomes ME and Reis RL, Cell encapsulation system using beads and fibres from natural origin polymers for cartilage tissue engineering applications, Termis Eu 2008 Annual Meeting, Porto, Portugal, June 2008

SECTION 1. BACKGROUND

Chapter I. POLYSACCHARIDES-BASED HYDROGELS USED FOR THE REGENERATION OF ARTICULAR CARTILAGE

Chapter I*
Polysaccharide-based hydrogels used for the regeneration of articular cartilage

ABSTRACT

This manuscript provides an overview of the *in vitro* and *in vivo* studies reported in literature focusing on seaweed polysaccharides based hydrogels that have been proposed for applications in regenerative medicine, particularly, in the field of cartilage tissue engineering. For a better understanding of the main requisites for these specific applications, the main aspects of the native cartilage structure, as well as recognized diseases that affect this tissue are briefly described. Current available treatments are also presented to emphasize the need for alternative techniques. The following part of this review is centered on the description of the general characteristics of algae polysaccharides, as well as relevant properties required for designing hydrogels for cartilage tissue engineering purposes. An in - depth overview of the most well known seaweed polysaccharide, namely agarose, alginate, carrageenan and ulvan biopolymeric gels, that have been proposed for engineering cartilage is also provided. Finally, this review describes and summarizes the translational aspect for the clinical application of alternative systems emphasizing the importance of cryopreservation and the commercial products currently available for cartilage treatment.

Keywords: Polysaccharide, hydrogels, cartilage regeneration, *in vitro* - *in vivo* application, clinical and commercial products.

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I.1. INTRODUCTION

Biological structures can be defined as open dynamic systems which interact and respond accordingly to changes of the environment. Therefore, to obtain a specific response from a target biological structure, it is necessary to create appropriate environments. A lot of work is being done to unravel the puzzle around structural and biochemical functions of the natural extracellular milieu that directs cell fate. Understanding the mechanisms of cell function, the typical response to generate matrix development and tissue growth, is critical for further advances in regenerating any damaged biological part. A great deal of research has been carried out developing biological constructs that support cell proliferation based on specific interactions between the biomaterial interface domains and the cell receptors to mimic the physiological environment. The natural extracellular matrix (ECM) is a hydrogel-like structure itself, comprised of several different biopolymers, encompassing a wide range of biological, chemical, and mechanical properties [1]. Generally hydrogels are used for cells growth and delivery, although being expected to behave like more than simple carriers with the goal of developing de novo tissues and ultimately regenerating and integrating the functional engineered tissue equivalent within the body. In the last few years, a vast range of different hydrogels that mimic more closely the native ECM have been proposed for regeneration strategies, produced by different methodologies and materials, with varying properties and composition. These hydrogels create a microenvironment for the cells to survive, multiply and produce ECM to form tissue substitutes. The knowledge obtained to this date, indicates that there is not a single ideal hydrogel available that can meet the requirements for all possible applications and thus, one must select a specific matrix with unique properties akin to target regenerative purposes. The use of polysaccharides as supportive systems for tissue formation reveals an increasing tendency in the biomedical field [2]. Among all the naturally derived polymers, the carbohydrate based polysaccharides, composed of sugar-ring building blocks, are emerging as a front runner in cartilage tissue engineering (TE) applications [3]. The polysaccharides of marine algae origin, especially the ones of seaweed source, such as alginate, agarose and recently κ-carrageenan or ulvan, which will be presented in this review, play important roles in biomedical applications aimed at maintaining the structure of the extracellular matrix, contributing with specific properties. Hydrogels based on polysaccharides are certainly the most studied structure type of biomaterials to heal and regenerate damaged cartilage, mostly because they resemble the dynamic ECM that constitutes this tissue [4]. In cartilage tissue, the cells are anchored into a matrix network which a hydrogel design intends to mimic. Thus, once embedded in hydrogels the cells renew and specialize due to spatial organization maintaining the round shape that characterizes specific cartilage cells phenotype.

Therefore, hydrogels that are morphologically similar to the native ECM, are commonly used as encapsulation systems [5], enabling the maintenance of chondrocyte phenotype similarly to the native tissue [6].

This review addresses the recent developments on the use of the above mentioned marine origin materials in studies envisioning the treatment and repair of cartilage defects. Several aspects are covered, namely the polysaccharide main characteristics/properties, *in vitro* and *in vivo* research and clinical/biomedical applications, with emphasis in cartilage TE (Figure 1).

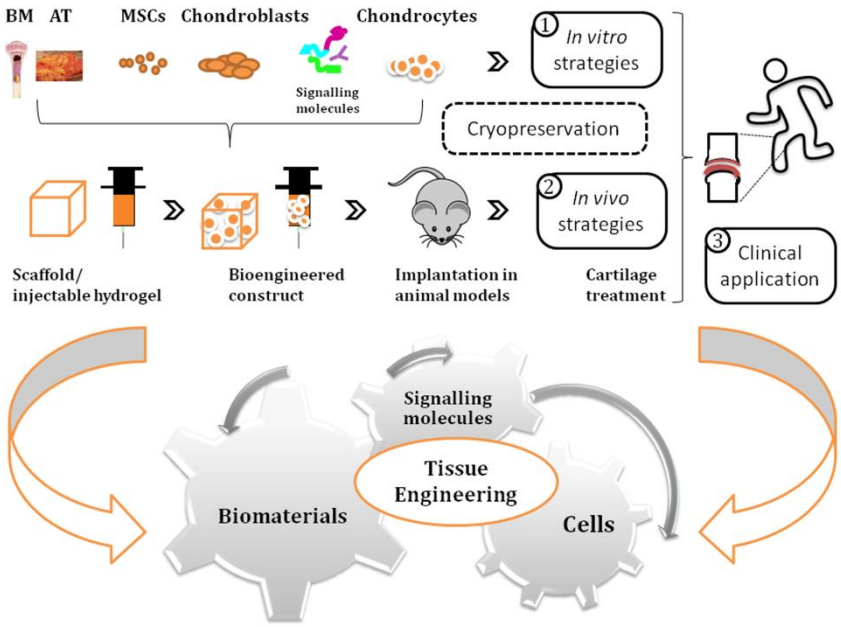

Figure I- 1. Schematic diagram showing multidisciplinary approach of cartilage tissue engineering (BM-bone marrow; AT-adipose tissue; MSCs-mesenchymal stem cells).

I.2. CARTILAGE TISSUE

I.2.1. Native cartilage tissue Structure and function

In order to design adequate therapies for the regeneration of damaged cartilage tissue it is essential to know and understand its structure, function, properties, so as to mimic as closely as possible cartilage native environment. Articular cartilage, found in joints, consists of a thin viscoelastic layer, usually less than 3 mm thick, which provides a smooth, near frictionless articulating surface [7]. Homeostasis of articular cartilage and the functions of chondrocytes depend on mechanical joint loading generated during daily activities [8, 9]. Cartilage tissue, is a stiff, dense and inflexible connective tissue without blood vessels, aneural and with few cells [10]. Chondrocytes represent only 1–5% of the articular

cartilage volume and also participate in the formation and maintenance of the ECM typically composed of collagens (collagen type II), large negatively charged hydrophilic proteoglycans (aggrecan), glycosaminoglycans (chondroitin sulfate, keratin sulfate) and smaller glycoproteins [11]. Their specific characteristics include no cell-to-cell contacts, spherical shape, high individual metabolic activity and ability to synthesize type II collagen. Furthermore the cells in articular cartilage receive nutrition through a double diffusion barrier, survive on low oxygen concentration and hence depend on anaerobic metabolism [12]. Details about composition with the corresponding ultra-structure can be found in Figure 2. Compared to other connective tissues, when cartilage is damaged the functional and metabolic properties of the original hyaline tissue will not be easily restored [13, 14]. In fact, considering the low cellularity and proliferative capacity of chondrocytes and due to specific characteristics such as being bound in lacunae with low migrating abilities to damaged areas [15] cartilage tissue underlies an intrinsic inability to repair. Given the limited spontaneous repair caused by the lack of vascularization preventing the onset of an inflammatory response following tissue injury, immediate and practical repair solutions are needed.

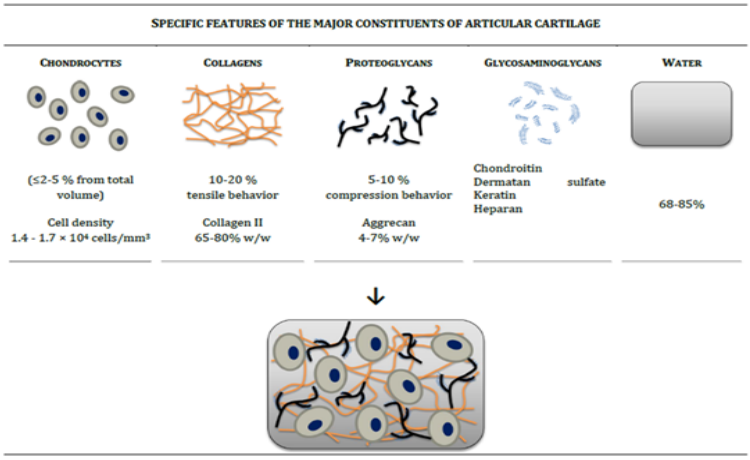

Figure I- 2. Schematic representation showing the structured organization of native cartilage tissue, as well as specific features of its main constituents, adapted [19].

I.2.2. Cartilage Pathologies

Damage of cartilage can be traumatic or degenerative and emerge as a result of a wide range of injuries or as an effect of another treated injury, having great impact on the quality of life of thousands of people. Factors such as obesity, alcohol abuse, and diabetes as well as mechanical factors like instability, trauma, and joint misalignment are causes for articular cartilage diseases [16].

Osteoarthritis and rheumatoid arthritis are examples of chronic conditions of cartilage damage. Defects can be associated to partial and full thickness extension to the underlying bone and, based on the macroscopic changes of the articular cartilage, are categorized in grade I, II, III and IV [17]. The increasing frequency of degenerative joint diseases caused by continuous aging and overweighted population and, due to high incidence of sports injuries, support the growing demands for the development of functional cartilage substitutes. More detail information on cartilage structure, diseases and treatment can be found elsewhere [18, 19].

I.2.3. Current repair strategies

Current strategies in the clinical field of cartilage repair progressed to what might be called the 3 "R" paradigm: reconstruction, repair, and replacement [20]. Cartilage regeneration strategies have evolved from marrow stimulation–based techniques to osteochondral transplantation and to cell-based repair techniques. Microfracture, the most common bone marrow-based technique, demonstrated positive short-term results. Nevertheless, after ~2 years, low integration and poor mechanical properties were evidenced [21]. Other available repair techniques that follow, based on autogenic (mosaicplasty) and allogenic tissue transplantation principles, showed excellent results for the first 5 years, but significant loss of viability upon 15 years [22, 23]. The therapeutic approach consisting in the autologous chondrocyte implantation (ACI), which include biologic replacement using a chondral biopsy taken from a donor site and cultured *in vitro* for a determined period of time, has been successful up to 10 years now [24, 25]. There are four generations of ACI therapies and all ultimately intend to preserve the chondrogenic phenotype, maintain cellular viability and function; the 3rd generation of ACI - matrix-induced autologous chondrocyte implantation (MACI) uses 3-dimensional (3-D) supports to achieve such requirements. MACI should secure the cells in the defect area, offer a more homogeneous chondrocyte distribution and minimize graft hypertrophy [26]. Critical issues that concern the above cartilage repair strategies consist of the donor tissue variability as well as the possible chondrocytes dedifferentiation. Alternative cell sources have been investigated, particularly allogenic adult mesenchymal stem cells (MSCs) [27]. A summary description of these cartilage repair techniques is detailed in Table I. Although these approaches offer good opportunities for the regeneration of cartilage defects, all current treatment options inflict some degree of tissue destruction before any therapeutic effect can be achieved. Consequently, the current available treatments based on "damage to heal approaches" [28], have limitations, which might be overcome through emerging TE strategies.

Table I- 1. Summary of clinical strategies for articular cartilage replacement, based on [24, 32].

Arthroscopic techniques		Cartilage replacement techniques	Cell based therapies	
Non reparative procedures	Reparative procedures – bone marrow stimulation		Autologous chondrocyte implantation (ACI)	Tissue engineering
debridement	Pridie drilling	mosaicplasty	First generation two stage procedure	Chondrocytes/ MSCs
chondral shaving	microfracture technique		Second generation collagen matrix	scaffolds or hydrogels
knee joint lavage	abrasion chondroplasty	osteochondral/chondral allografts	Third generation MACI	GF/ cytokines
			Fourth generation MSCs	

MACI - Matrix-induced autologous chondrocyte implantation, MSCs - mesenchymal stem cells, GF - growth factors.

I.3. CARTILAGE TISSUE ENGINEERING

As suggested above, the development of biological tissue substitutes through TE, may provide advantages over the current repair procedures enabling alternative therapies and hope for cartilage defects regeneration (Figure 1). These potential advantages include the formation of a more reliable hyaline cartilage tissue, through the delivery of appropriate cell types embedded in a suitable hydrogel [29] promoting enhanced integration with surrounding tissues [30]. However, to achieve further improvements, minimally invasive procedures and innovative cell carrier concepts should be refined [31].

Furthermore, there is a lot of debate over which is the most suitable cell source to be used in cartilage cell-based therapies. Ideally, a cell source should be easily available, enable an excellent yield number, high proliferation capacity, stable phenotype/genotype, no issues of immunogenicity or disease transmission risks, and no donor site morbidity. For cartilage TE, most of the published studies refer to the use of primary autologous chondrocytes [32, 33] and adult stem cells [34, 35]. Although chondrocytes are an obvious choice as a source of cells for cartilage TE, a number of disadvantages [36, 37] have led to intense interest in alternate cell types that can differentiate into the chondrogenic lineage. Bone marrow-derived stem cells (BMSCs) [38] and adipose tissue-derived cells (ASCs) [39, 40] are frequently considered top candidates for cartilage TE, due to their ability to create functional cartilaginous tissues. Some advantages and disadvantages of the two stem cell sources may be clearly identified in terms of collection procedures, cell number and proliferation capacity (Table II). Some

studies described a higher chondrogenic potential of BMSCs when compared to ASCs [41] likely due to their role in bone formation, including endochondral ossification.

Another important issue is to determine the optimal number of cells (chondrocytes or stem cells) for a successful *in vivo* application. Although this is still under consideration, several studies suggest that higher cellular content induces better tissue repair [42-44]. Commercially available sources of chondrocyte suspensions recommend a dose between 0.5 and 2.0 × 10^6/cm^2 and similar cell values (2 to 3 × 10^6 for a 4- cm^2 collagen membrane) have been used in the clinical practice [45]. Moreover, the chondrocytes density in native cartilage tissue is around 1.4 ×10^7 cells/cm^3, i.e., about 5-10 % of the cartilage volume, but such cell density is difficult to replicate *in vitro* due to slow growth rates that differ depending on the cell type [46]. The process of chondrogenesis is regulated by a number of growth factors including the members of transforming growth factor beta family (TGF-β, BMPs- bone morphogenetic proteins) [47]. The *in vitro* experiments have confirmed that growth factors supplementation enhance the production of cartilage in tissue-engineered constructs [48, 49]. Clearly chondrogenesis is a complex process which involves not only biological growth factors, but also a carefully controlled time dependency [50, 51]. Furthermore, this process can be dramatically influenced by the 2D and 3D (two and three dimensional) environment in which cells are delivered /cultured. The fact that hydrogels provide 3D cellular microenvironments that can be tailored to stand physical, chemical and biological signals, has encouraged the development of engineered functional tissue equivalents based on such systems.

Several natural origin hydrogels sources including proteins (collagen, elastin, silk fibroin, fibrin), polysaccharides (chitosan, chondroitin sulphate, hyaluronic acid) and seaweed source (alginate, agarose, carrageenan, ulvan) have been extensively studied for cartilage repair [52]. According to data collected from the literature, the reasons for using seaweed polysaccharides in regeneration applications lie in their intrinsic features, such as chemical similarity with native tissue components, non-harsh processing, variable degrees of hydrophilicity and biocompatibility [53]. Natural materials obtained from algae are often preferred for biological applications since they are believed to elicit low immune response when choosing a potential biological application [54]. Most of them offer advantages concerning biocompatibility, which is of extreme importance for the integration with the surrounding tissues. Moreover, they are readily available, inexpensive and easy to fabricate into hydrogels, making them appealing choices for designing hydrogels to be used in different biomedical application [3]. In addition, the hydrogel structure protects the entrapped cells against the immune system of the host, simultaneously allowing the unhindered passage of nutrients, oxygen and secreted therapeutic factors

or proteins [55]. The hydrogels design can vary from injectable systems to solid structure like discs, fibers, cylinder, and capsules ranging from micro to macro dimensions.

Table I- 2. List of supporting and opposing arguments in applying stem cells versus somatic, precommitted cells for cartilage tissue engineering.

CELL SOURCE	PRIMARY CULTURE	STEM CELL		REF.
	CHONDROCYTES	*BONE MARROW (BMSCs)*	*ADIPOSE TISSUE* (ASCs)	
Collection procedures	Cartilage biopsy	Marrow aspirate	Liposuction or reconstructive surgery	[45]
Isolation site influence	donor site morbidity & contamination or potential infection	more invasive, painful and not frequently result of another intervention	performed in parallel with another surgery, safe and easy to obtain	
Cell yield (number)	limited	limited numbers, quickly amplified in monolayer	high 400000 stem cells/ml adipose tissue	[60]
Mitotic potential	slow	medium	high	
Self renewal capacity	low	limited	high	
Plasticity	tendency to dedifferentiation	dedifferentiation is not an issue	dedifferentiation is not an issue	[61]
Isolated tissue origin	provoke immune rejection	supportive cells for haematopoiesis, exists 1 out of every 105 cells	not an issue	
Additional procedures	no need of GF or can use GF to not dedifferentiate (TGF-β3)	TGF-β1, TGF-β3, BMPs	TGF-β1, TGF-β3, BMPs	[62]

BMSCs – bone marrow mesenchymal stem cells; ASCs – adipose derived stem cells, Growth factors- GF; transforming growth factor β - (TGF-β); Bone Morphogenetic Proteins - (BMPs)

I.4. ALGAE POLYSACCHARIDES – DETAILED CHARACTERISTICS

Some algae polysaccharide based hydrogels, namely alginate and agarose have already been extensively studied for TE applications, but others such as carrageenan and ulvan are just starting to be investigated. Even though hardly applied in the field of cartilage repair, these algae polysaccharide have recently registered an increased attention in the biomedical research field. Such consideration is owed to the intriguing feature of the amounts of sulphate found in their structure, whose beneficial biological properties prompt scientists to focus on their use in the biomedical fields [56]. Certainly the presence of sulphate groups in their structure/composition and the chemical affinity with mammalian glycosaminoglycans (GAGs) due to the similar chemical content play important roles in the antiviral, anticoagulant, antioxidant and anticancer activity of these polysaccharides [57].

The carrageenans are temperature dependent materials since they are soluble in water above 60 °C and gel upon cooling to temperatures between 30 and 40 °C being designated as a physical hydrogels. The gelation of carrageenan is induced by the reversible temperature sensitive formation of intermolecular hydrogen bonds and involves a coil to helix conformational transition followed by helix aggregation [58]. The thermoresponsive solubility behavior and the gelation promoted by monovalent cations such as potassium ions open perspectives to develop systems that gel at body temperature [59]. They are characterized as high molecular weight polysaccharides with high sulfate-ester content, meaning higher level of solubility and lower gel strength [60]. Ionically cross-linked hydrogels, such as carrageenan, normally undergo slow dissolution that can be shifted through chemical modification and the stiffness can be altered as well, allowing tuning of the mechanical properties [61]. Ulvan structure shows great complexity and variability as evidenced by the numerous oligosaccharide repeating structural units identified [62]. The main constituents of ulvan are sulfated rhamnose residues linked to uronic acids, resulting in a repeated disaccharide unit β-D-glucuronosyl-(1,4)-α-L-rhamnose 3-sulfate, called aldobiouronic acid [63]. In aqueous solution, ulvan tend to form microaggregates due to the polymeric material not fully dispersed indicating limit number of functional groups available for chemical modifications thus, hampering its potential versatility [64].

The overview on the friendly gelation mechanism induced by temperature or ions clearly demonstrates the relevance of the use of such biopolymers in the field of TE. In particular sulphated polysaccharides (carrageenan and ulvan) present a real potential for delivering products for therapeutic applications providing a valid alternative to mammalian glycosaminoglycans. More details regarding seaweed type, gelation or degradation mechanism and the interactions of alginate, agarose, carrageenan and ulvan

with cells are summarized in Table III. For complete description of chemical structure, gelation process or other characteristics of algae polysaccharide consider consulting existing publications [56, 65, 66].

Table I- 3. Important properties of algae polysaccharide (modified from [6, 75]).

POLYSACCHARIDE	SEAWEED TYPE	GELATION	DEGRADATION	CELL INTERACTION	REF.
Alginate	Brown Seaweed	Ionic (Ca^{2+})	Ion exchange; others	Low	[76]
Agarose	Red Seaweed	Thermal	Non-degradable	Low	[77]
Carrageenan	Red Seaweed	Thermal & Ionic (Ca^{2+}/K^{+})	Ion exchange	Low	[78]
Ulvan	Green Seaweed	Ionic (boric acid and divalent cations)	Enzymatic degradation	n.d.	[79]

n.d. – not determined

However, not every characteristic comes only as an advantage. Table IV summarizes pros and cons features reported for the polysaccharide hydrogels. For example, the lack of manufacture reproducibility on a large scale, batch-to-batch variability and also the presence of impurities in the final processed material due to the extraction process, constitute major concerns [67]. Recent data suggests that contaminants during extraction processing are likely to cause an immune response but this issue is still being disputed, although such molecules can be removed by purification methods [68, 69]. These are time- and money-consuming processes and introduce variation in the biological response. This factor is far more important than holding mechanical properties and expecting that the synthesized cartilaginous tissue growth acts as reinforcement [70].

Other associated common weakness of the seaweed based hydrogels are related to inadequate mechanical properties, which is a common trend [71]. Nonetheless, it is expected that a hydrogel structure will partially tolerate shock absorption and deformation, mimicking articular cartilage characteristics. Enhanced mechanical properties can be achieved by adjusting various parameters, including the concentration, the crosslinking density and the possibility of mixing such hydrogels with composite. In addition, introducing photo-crosslinkable parts, appropriate to the chemical structure of

the hydrogels, can modify the stiffness of the structure but simultaneously compromise the viability of the cells [72].

In terms of cellular response, these natural algae polysaccharide have been investigated as potential delivery matrices based on encapsulation techniques, for cell transplantation [73]. Their gelatinous consistency enables ''anchorage independent'' 3D cell immobilization, namely i*n situ* hydrogel formation, which facilitates subsequent cell differentiation [74]. Like in many hydrogels, there is no integrin interaction between cells and the hydrogels matrix, therefore, cells retain their rounded shape, likely to enhance chondrogenesis [75]. On the other hand, long-term integrin binding can lead to dedifferentiation and formation of fibrocartilage [75]. Furthermore, hydrogels being hydrophilic and binding to water provide few sites for the cells to attach, directly affecting cell viability and proliferation.

Table I- 4. Main advantages and disadvantages of carbohydrate polysaccharide hydrogels from algae origin.

POLYMER NAME	ADVANTAGES	DISADVANTAGES	REF.
Alginate	☑ Low toxicity ☑ Cheap ☑ Easy to gel	☒ Degrades through ionic exchange with surrounding media ☒ Minimal cellular interaction ☒ Protein not easily absorbed	[160, 161]
Agarose	☑ Easy to control pore sizes ☑ Easy to gel	☒ Larger pores ideal for cellular growth introduce structural instability ☒ Low mechanical properties	[162, 163]
Carrageenan	☑ Thermally reversible ☑ Cheap ☑ Easy to gel & manipulate ☑ Display close similarity with mammalian glycosaminoglycans	☒ Degrades through ionic exchange with surrounding media ☒ Influenced by temperature, pH and cations concentration	[164-166]
Ulvan	☑ Abundant and cheap source ☑ Great chemical functionalities ☑ Display close similarity with mammalian glycosaminoglycans	☒ Contain proteins which need specific purification protocols ☒ Formation o microaggregates when dissolved in water	[64]

I.5. IN VITRO APPLICATIONS OF HYDROGELS BASED ON ALGAE POLYSACCHARIDE

Considering all the above mentioned polysaccharide hydrogels characteristics, this section will review some of the most extensively studied algae polysaccharide-based hydrogels, such as alginate and agarose and some new candidates, like carrageenan and ulvan, for the culture and differentiation of stem cells and chondrocytes. In what follows, it will be provided an overview of different approaches and parameters including hydrogel concentration, different cell source and cell number, as well as the stimulation with various growth factors, envisioning application in cartilage regeneration. Table V summarizes the most recent studies concerning cartilage TE applications using algae polysaccharide hydrogels.

I.5.1. Alginate

Alginate continues to be the most widely used hydrogel for *in vitro* studies due to its easy production, effectiveness and low cost (Tables III and IV). Applications of alginate in the cartilage TE field revealed that after an initial cell loss, chondrocyte maintained their typical chondrocyte phenotype [76]. To maintain the chondrocytic phenotype and the synthesis of ECM proteins, alginate has been used *in vitro* as a matrix for the three-dimensional culture of human articular chondrocytes from elderly patients [77]. Similar performance was reported in another study where human articular chondrocytes, embedded in alginate beads, showed enhanced collagen type II and aggrecan expression [78, 79]. Moreover, the 3D alginate culture system was proven to be efficient in keeping high viability, chondrogenic phenotype and promoting the redifferentiation of articular chondrocytes [80, 81]. A different work show that chondrocytes encapsulated in alginate maintain their viability and function due to the addition of microchannels to the polymeric hydrogels [82]. In another study, the supportive alginate-based hydrogels provided an environment and ways to deliver chondrocytes and compared with monolayer culture, stimulated the deposition of sulphated glycosaminoglycans (sGAG) and collagen type II, although without promoting redifferentiation [83]. Furthermore, alginate hydrogels have been used to expand and induce stem cell differentiation [84]. Human MSCs encapsulated in alginate beads undergo chondrogenesis, demonstrated by the cells assuming a rounded morphology with lacunae. Hyaline cartilage-like tissue was developed, showing to be positive for Safranin-O staining and immunohistochemistry of typical chondrogenic markers, namely COL2A1 and COL10A1 [85]. Human MSCs showed a time-dependent accumulation of sGAG, aggrecan and type II collagen in this type of hydrogel [86]. In other study, bone marrow-derived stem cells (BMSCs) encapsulated in alginate evidenced enhanced cartilaginous matrix accumulation over agarose [87]. Alginate demonstrated to

improve the stability of the system, supporting sGAG and collagen II production as well as chondrogenic gene expression, when human BMSCs were entrapped in a blend that included fibrin in addition to alginate [88]. Induction of chondrogenesis of stem cells isolated from adipose tissue in alginate hydrogels has also been achieved. After 2 weeks of *in vitro* culture the adipose-derived stem cells produced cartilage matrix proteins which show dependency of physical environment and culture conditions [89]. To overcome weaknesses like low mechanical and uncontrollable degradation properties, ionically cross-linked alginate hydrogels have been modified, with no toxic effects on the encapsulated MSCs, supporting chondrogenic differentiation [90]. Recently, investigators have produced hybrid constructs mixing alginate with other materials or tailored alginate with synthetic adhesion peptides as ways of improving its properties [91-93].

I.5.2. Agarose

Agarose has also been widely investigated in cartilage TE strategies (Table V). For example, encapsulation within agarose hydrogels has shown to support rabbit articular chondrocytes redifferentiation [94]. Articular chondrocytes seeded on agarose hydrogels demonstrated enhanced chondrogenic matrix elaboration when applied under physiological deformational loading, accelerating the formation of a cartilage-like tissue [95]. Compressive mechanical forces are transmitted to the embedded chondrocytes which respond by producing extracellular matrix proteins leading to increased stiffness of the newly developed engineered tissue [96]. The culture of murine chondrocytes embedded within agarose hydrogels has shown to maintain the chondrocytic phenotype, allowing matrix deposition around chondrocytes and showing that chondrocytes sense and respond to mechanical forces [97]. The concept of developing functional TE systems centered on the use of bioreactor enabling physiologic-like loading, has also reported successful results with immature bovine primary chondrocytes but did not produce the same outcome when using adult canine primary chondrocytes [98]. Moreover, studies suggest that the age of the cells plays an important role, showing that unpassaged cells can elaborate inferior matrix as compared to passaged mature chondrocytes. The continuous supplementation of TGF-β3 in combination with mechanically loading prior to implantation also seems to result in an improved engineered tissue substitute [99]. Agarose hydrogels have also been investigated in combination with mesenchymal stem cells, including human adipose-derived stem cells and bovine mesenchymal stem cells, for a variety of applications, including cartilage repair, as shown in Table V. Agarose hydrogels are noted for their ability to promote and maintain the chondrogenic phenotype of bone marrow stem cells (BMSCs), with deposition of cartilage-specific

biomacromolecules [100]. Another work showed that the chondrogenesis of the stem cells was directly correlated with the number of cells used, meaning that higher cell density led to enhanced expression of cartilage-specific gene [101]. It has also been reported that BMSCs produced an ECM with lower mechanical properties than that produced by differentiated articular chondrocytes embedded in agarose hydrogel [102, 103]. Findings reported in other publications such as the induction of chondrogenesis in hMSC-seeded agarose constructs without TGF-β suggest that, application of hydrostatic pressure may initiate chondrogenesis faster than lower pressure [104]. Although, the exclusion of TGF-β3 from culture conditions has been reported to have significant effect on the mechanical properties and also on the biochemical content, the functionality of cartilaginous tissues using MSCs from joint infrapatellar fat pad, encapsulated in agarose hydrogels, has resulted in robust chondrogenesis, as shown by another study [105].

I.5.3. Carrageenan

Carrageenan hydrogels have been mostly used as drug or growth factor delivery systems [106-108], immobilization of enzymes [109] and in pharmaceutical formulations [110]. However, the characteristics and specific properties of these natural derived hydrogels, concerning their potential for cartilage regeneration are poorly exploited in the literature [111]. Upon adding cations, the carrageenan solution rapidly forms a gel and can be used as in situ cell matrix delivery material, due to its mild cross-linking properties [112] which makes it extremely interesting to be applied in the biomedical field [113]. Nevertheless, carrageenan has been used for the encapsulation of human-adipose-derived stem cells (hASCs), human nasal chondrocytes (hNCs), and a chondrocytic cell line (ATDC5) showing potential for cartilage regeneration strategies [114]. The findings from this study showed that hASCs embedded in κ-carrageenan hydrogels exhibited higher expression of typical cartilage markers (collagen type II and aggrecan) than chondrocytes providing further evidence for the advantageous use of hASCs as an alternative cell source in cartilage treatments (Table V). In a subsequent study, hASCs encapsulated in κ-carrageenan hydrogel and cultured with medium supplemented with chondrogenic growth factors appeared to increase the stiffness and the viscoelastic properties of the hydrogel construct providing a versatile platform for cartilage TE [115].

I.5.4. Ulvan

Ulvans are the major constituents of green seaweeds cell walls (Ulvales, Chlorophyta) and are composed of rhamnose, glucuronic acid, iduronic acid, xylose and sulphate [65]. These green algae

can be prepared into different structure designs such as membranes [116, 117], particles [118], hydrogels [119] and 3D porous structures [117]. Due to the difficulties in identifying the chemical structure of algal sulfated polysaccharides, the relation between their structures and biological activities is not completely understood [120]. Despite ulvan chemical variability, biological properties have been frequently reported like antioxidant effects [121], antitumoral activity [122], immunostimulating ability [123]. Antihyperlipidemic activities [124] or antiviral effects [125] have also been studied. Subsequent to all of these findings, ulvan polysaccharides are considered to have great potential for biomedical applications (Table V). Nevertheless, relevant studies on ulvan are limited and their applicability may range from drug delivery [117] to wound dressing or TE [118, 126].

Table I- 5. A summary of key studies from the current literature describing polymers hydrogels, cell types and cell densities used in laboratory cartilage tissue engineering applications

POLYMER CONCENTRATION	CELL SOURCE	SIGNALING MOLECULES	CELL DENSITY (cells/mL)	REF.
ALGINATE				
1.2 % (w/v)	Human chondrocytes	-	2×10^6	[100, 102, 146]
20 mg/mL; 2 % (w/v)	Non human chondrocytes	IGF-1, TGF- β1	$25\text{-}50 \times 10^6$	[146-149]
20 mg/mL	Human bone marrow MSCs	TGF-β3	20×10^6	[104, 106]
2 % ; 1.5 % (w/v)	Non human bone marrow MSCs	TGF- β1 or Dex;	20×10^6; $1\text{-}2 \times 10^6$	[105, 150]
2 %; 1.2 % (w/v)	Human adipose tissue MSCs	TGF- β1; BMP-6	10×10^6; 5×10^6	[151, 152]
AGAROSE				
2 % or 3 % (w/v)	Non human chondrocytes	TGF- β3	$30 - 60 \times 10^6$	[32, 44, 115]
2 % (w/v)	Non human bone marrow MSCs &	TGF- β3 or -	$10 - 60 \times 10^6$	[115, 118-120, 153-155]

	chondrocytes			
2 % (w/v)	Human bone marrow MSCs	TGF- β3	3, 6, and 9 × 10^6	[118, 120, 121]
2 % (w/v)	Human adipose tissue MSCs	TGF- β1	10 × 10^6	[151]
2 % (w/v)	Non human bone marrow & adipose MSCs	TGF- β1; TGF- β3	10 - 15 × 10^6	[156], [46, 118, 157-159]
CARRAGEENAN				
0.8 % and 1.2%	Human chondrocytes	-	2 × 10^6	[160]
1.5 % (w/v) 2.5 % (w/v)	Human adipose tissue MSCs; Human nasal chondrocytes;	TGF- β1	5 × 10^6	[161, 162]
2 % (w/v)	ATDC5-chondrocytic cell line	-	1 × 10^6	[129]
ULVAN				
5 %; 8 % (w/v)	L929-mouse fibroblasts	-	5 × 10^5 cells/ structure	[145]

GF - Growth factor; TGF - Transforming growth factor; BMP-6/ BMP-2 - Bone morphogenetic protein - BMP-6; Dex - dexamethasone; IGF-1 - Insulin-line growth factor; ES - embryonic stem; ATDC5 - mouse teratocarcinoma AT805 derived cell line

I.6. IN VIVO APPLICATIONS OF HYDROGELS BASED ON ALGAE POLYSACCHARIDE

It has been noted that even though research carried out *in vitro* provides high quality outcome, it usually serves as a precursor to more complex *in vivo* methods. As a first step in demonstrating the *in vivo* chondrogenic potential of tissue engineered constructs, investigators have used heterotopic animal models, typically consisting on the implantation of the bioengineered cartilage tissue in dorsal subcutaneous pouches of immunocompromised nude mice [127]. Although these experiments may indicate the biological construct performance under *in vivo* conditions, different results may be observed when the cartilage substitute is implanted into a cartilage defect.

I.6.1. Alginate

Several i*n vivo* studies have been conducted aiming at assessing the potential of alginate as a supportive matrix for different relevant cell types, including primary chondrocytes and stem cells from different origins, in various animal models. Alginate seems to stimulate chondrogenesis as suggested by studies where chondrocytes were seeded and stimulated to produce a cartilage-like matrix, being subsequently implanted subcutaneously in nude mice for 8 weeks [128]. The same study suggests that the addition of alginate provided retention of the cartilage graft shape without any influence on the amount of cartilage matrix proteins produced per tissue wet weigh. The implantation of alginate with allogenic rabbit chondrocytes, followed up until 6 months, provided complete *in vivo* reconstruction of a full-thickness articular cartilage defect [129]. After 6 months, the implantation of alginate alone developed only fibrous cartilage while the suspension of chondrocytes led to the recovery of a normal tissue structure. On the other hand, transplanted chondrocytes in an alginate gel implanted into full-thickness osteochondral defects in rabbits didn't form repaired tissue and cells decreased in number with time [130]. The evaluation of chondrogenesis of human adipose tissue-derived stromal cells when cultured in alginate hydrogels and implanted subcutaneously in nude mice for up to 20 weeks [131], reveled significant production of cartilage matrix proteins, suggesting the beneficial use of alginate systems for cartilage regeneration, as they maintain stable cartilaginous phenotype with no sign of hypertrophy during 20 weeks. Also, using a rabbit model, full-thickness defects filled with alginate beads containing rabbit stromal cells remained viable, showing chondrogenic phenotype embedded in a positively stained proteoglycans matrix and occupying the defects within regenerated tissue [132].

I.6.2. Agarose

Agarose hydrogels laden with different cells types have been studied to assess their ability to induce the *in vivo* development of cartilage tissue. One of these studies showed that, 18 months after the transplantation of allogenic chondrocytes in agarose hydrogels in rabbits, neo cartilage tissue was formed, exhibiting higher type II collagen, proteoglycan contents when compared with untreated defects. Additionally, control implants of agarose alone produced poor fibrous substitute tissue, insufficient healing and incomplete filling of the cartilage defects [133]. In another study, the implantation of chondral and osteochondral constructs based on primary or passaged (using growth factors) canine chondrocytes encapsulated in agarose showed no gross or histological signs of rejection and excellent integration with surrounding cartilage and subchondral bone [134]. However, when agarose constructs were seeded with stem cell and subcutaneously implanted in nude mice, they

showed significant decrease of sGAG content, while no significant change was observed using primary chondrocytes, indicating that the *in vitro* generated chondrocyte-like phenotype was transient [135].

Table I- 6. A summary of key studies describing polymers hydrogels, cell types, densities and implantation time register in experimental animal models for cartilage tissue engineering applications

POLYMER CONCENTRATION	CELL TYPE	FORMULATION CELLS DENSITY	ANIMAL MODEL (DEFECT)	FOLLOW UP TIME	REF.
ALGINATE					
2 % sodium alginate; 1.2 %; 0.75 %	Chondrocytes	10-50 × 10^6 cells/mL	subcutaneous implantation, mice; defect in rabbits;	8 weeks; 30 weeks; 6 months	[164, 171]
2% alginate; 1.2 % alginate beads	Chondrocytes	50 × 10^6 cells/mL; 10^7/ml; 4 × 10^6 cells/mL	subcutaneous implantation, sheep/ nude mice; defects 4 mm diameter mini-pigs	1 week; 5 weeks; 6 months;	[172-174]
1.2 % ; 2 % sodium alginate	Bone marrow MSCs	1× 10^6 cells/defect	Ø3×6 mm defects in rabbits; Ø4×5 mm defects in rabbit	3 months	[167, 175]
2.2 % barium alginate; 1.25 % sodium alginate	Human & mouse ESCs	10 × 10^6 cells/mL	SCID and BALB/c nude mice		[176, 177]
AGAROSE					
2 %	Chondrocytes	-	rabbit	18 months	[168]
2 %	Chondrocytes	30 × 10^6 cells/mL.	canine knee	12 weeks	[169]
0.6 - 1 %	Periosteum	-	rabbit knee	6 weeks	[178]
0.6 %	Human bone marrow/ adipose tissue MSCs	10 × 10^6 cells/mL	injection, muscles of athymic mice	5 weeks	[179]

2 %	Bone marrow MSCs	20×10^6 cells/mL	subcutaneous implantation, nude mice	28 days	[170]

MSCs – mesenchymal stem cells; Ø – diameter; SCID - albino mouse strain with spontaneous severe combined immunodeficiency mutation; BALB/c - albino, immunodeficient inbred mouse strain; ESCs – embryonic stem cells;

I.7. FROM LABORATORY TO CLINICAL APPLICATION

Although the *in vivo* trials involving cell laden hydrogels have registered different outcomes, mostly depending on the cell type used and cell number, the polymer concentration tested and the size of the defect treated, it is clear that overall, the use of hydrogels in cartilage regeneration strategies produces positive results.

Nevertheless, there have been few clinical studies on products applicable to humans, which mean that engineering cartilage repair strategies still require improvements before they get into a standard clinical application and commercialization. The clinical approach to any cartilage repair technique should be customized based on both patient-specific and lesion-specific factors [20]. The engineered graft has to fit completely to the damaged area, providing support and biological cues to restore the tissue function. A well-designed clinical model to regenerate cartilage should always consider the safety, efficiency and simplicity of the developed system, even if it is necessary to compromise the quality of the regenerated tissue [13]. Additionally, to translate research-scale production into routine clinical use, biological cartilage substitutes will need to demonstrate cost-efficiency ratios that are beneficial over other existing treatments and excellent reproducible results in order to perform reliable quality control and standardization [136].

One important development for the translation of TE products into the clinical/industrial scenario would be the production of "off the shelf" products, eliminating the waiting time and reducing patient incapacity period, relying on the availability of the engineered grafts upon immediate clinical need. This could be achieved by common cryopreservation approaches that may assure the tissue substitutes functionality ultimately shortening the time of developing engineered constructs set up on cell culture procedures [137, 138]. This would be a way to specifically design, generate and scale up, ready-to-use engineered tissue substitutes guaranteeing immediate accessibility for clinical applications. However, few studies have focused on tissue substitutes banking and storage technologies, addressing the impact of such process on hybrid constructs that contain a scaffold/carrier material and cells [137, 139]. Yet, there are studies that have reported the effect of cryopreservation in cell encapsulating systems (hydrogels) [140, 141] using several different cryoprotectants, [142, 143] and vitrification

solutions [138, 144], predicting that tissue-engineered products can be cryopreserved without prejudice of their functionality.

It is already possible to find several hydrogels approved or under clinical trials for cartilage treatments (see Table VII). The most well-known commercial product based on polysaccharides is a hybrid medical grade agarose-alginate hydrogel, Cartipatch® (Company/Responsible - TBF Genie Tissulaire). This product was tested for implantation of autologous chondrocytes in chondral and osteochondral human defects. After a minimum follow-up of two years, patients with lesions larger than 3 cm^2 improved significantly more than those with smaller lesions. Also, in 8 out of 13 patients, hyaline cartilage-like repaired tissue was predominantly observed [145]. In a phase III clinical trial alginate gel suspension (Company/Responsible - Curis, Inc.) was used injecting chondrocyte harvested from the ear cartilage of the patient for the treatment of pediatric patients with vesicoureteral reflux. As seen in Table VII the majority of commercial tissue engineering products developed for cartilage regeneration are protein collagen-based hydrogels, since they are a component of ECM and due to the presence of bioactive domains in its structure regulates important process during chondrogenesis [146]. However, other clinical products are being used to promote cartilage repair namely, hyaluronate (hyaluronic acid) or synthetic polymers. Although there is an extensive range of products for cartilage treatment, only few of them are approved for clinical trials, the rest are still in different phases of clinical use.

Table I- 7. Commercial products in cartilage tissue engineering adapted from [190-192].

PRODUCT NAME	COMPANY	PRODUCT COMPOSITION	REGULATORY STATUS	FORM & USE	REF.
CARTIPATCH	Tissue Bank of France, Lyon, France	agarose-alginate hydrogel & autologous chondrocytes	clinical trial phase is unclear	chondral and osteochondral	[189]
CARTICEL, MACI	Genzyme Biosurgery, Massachusetts, USA	type I /III collagens (porcine) & autologous chondrocytes	completed phase III clinical trials; product not available	sheet- articular cartilage injury	[193-195]
ATELOCOLLAGEN	Koken Co. Ltd, Tokyo, Japan	type I collagen	clinical trial phase is unclear		[196, 197]

CaReS	Arthro Kinetics, Esslingen, Germany	type I collagen (rat-tail) & autologous chondrocytes	2007 Germany;	3D discs – articular cartilage injury	[198]
Novocart 3D	TETEC Tissue Engineering Technologies AG, Reutlingen, Germany	collagen-chondroitin-sulfate based membrane & autologous chondrocytes	clinical trial phase is unclear		[199]
Hyalograft® C	Fidia Advanced Biopolymers, Abano Terme, Italy	benzylic ester of hyaluronic acid (roosters) & autologous chondrocytes	commercial sale began in 2000; completed phase III clinical trials	3D discs – articular cartilage injury	[200-202]
BioSeed-C	Biotissue Technologies, Freiburg, Germany	polymer of polyglycolic/ polylactic acid and polydioxanone & autologous chondrocytes	First clinical trials were in 2001; completed phase II clinical trials	3D discs – articular cartilage injury	[203, 204]

Status and descriptions of the clinical trials are available from the web site of U.S. National Institutes of Health, http://clinicaltrials.gov/.

I.8. Summary and future directions

Research on polysaccharide-based hydrogels for TE application is continuously improving and there are many questions that instigate the attempts to regenerate cartilage. To mimic the structural (biochemical and histological characteristics) and functional (compression properties) complexity of native cartilage is a challenging objective to accomplish. Thereby, every attempt to achieve this aim brings scientists closer in the process of assembling the necessary knowledge to repair damaged cartilage tissue.

The knowledge and research so far supports that natural cell-carrying matrices based on algae polysaccharide are suitable for engineering cartilage. Many of these hydrogels are combining well with the cells and can be designed as injectable systems, setting *in situ* and filling any shape and size of the

recipient defect. All of these available data needs to be compiled in order to develop clinical relevant solutions for cartilage regeneration, considering that the functionality of these hydrogels in the laboratory and animal experiments had been established.

While *in vitro* the developed tissue engineered constructs show promising results, the potential value for *in vivo* treatment of cartilage defects remains uncertain, as setup parameters of the performed *in vitro* and *in vivo* studies vary to a great extent. Furthermore, i*n vitro* manipulation of either chondrocytes or stem cells used is required in most of the currently available approaches. In fact, culturing the cells prior to *in vivo* implantation allows creating a mature cartilage and the presence of matrix around the cells is known to enhance donor cell retention at the repair site [21]. However, the manipulation of the cells *in vitro* increases concerns on how cells may be affected and increases the complexity of the treatment, compromising the safety of the procedure and the associated cost. Despite the advantages of using polysaccharide hydrogels in cartilage TE, some concerns remain which result mainly from insufficient characterization of the cell hydrogel construct or the level of repaired tissue formation and from too short follow up periods. Moreover, the cell culturing conditions, cell number, as well as the cell origin, leads to varied results and finally serious concern in estimating the potential clinical application of the tissue substitute (Tables V and VI). Findings have demonstrated that cell density is likely a key parameter to consider in TE design, since cells could not develop cell-cell contacts or express cartilage like tissue component without some minimal cell density which can vary with the source of cells [147]. Also, cells respond differently to substrate rigidity therefore the increase in polymer concentration, or stiffness involves consequences on the cell morphology, cytoskeletal structure, and on stem cell differentiation [148, 149]. Ultimately, standardized procedures for cells, media and growth factors are required anticipating that in the future, more detailed studies will elucidate the role of cell density or bioactive molecules hold in chondrogenesis. Although current cartilage TE strategies investigate a wide range of hydrogel materials in combination with a large variety of cell source, the existing data demonstrate that the optimal hydrogel type has not been determined so far, while outcomes also depend on the cell type - stages of differentiation, culture conditions [150]. Nevertheless, hydrogels based on marine origin polysaccharide are still commonly used and each individual natural polymer has strengths and flaws to its use and results can vary depending on the application.

Besides these comments, consideration should be given to the lesion location and damage size, activity level and patient age. These are influencing parameters in choosing the right cartilage repair techniques and controlling the outcome of the treatment. More clinical trials are needed to find definitive answers and to develop procedures that relieve patient pain and produce a durable replacement for damaged cartilage. Up until the present day, no treatment has been shown to

completely regenerate or restore articular cartilage/ subchondral bone to a normal status, therefore articular cartilage repair remains under intense investigation and the cure is yet to be defined. The newer generation of repair/regeneration techniques has shown some promise, but long-term outcome is still unknown.

Furthermore, the extended variety of existing polysaccharides and their inherent features opens a wide range of opportunities for synergistic fabrications of new multifunctional biomaterials [151, 152]. The development of strategies aiming to overcome hydrogels limitations is gaining increasing interest. Such alternative encompass the fabrication of blended systems, or chemically and/or physically modification of their original structure. Modifying the hydrophobic properties of the polysaccharides to self-associate, reducing the molecular weight by depolymerization and changing the sulfation content are ways to improve polysaccharide-hydrogels properties [144]. The addition of interest signaling molecules is another way to confer a better outcome performance to the overall tissue engineered constructs [153].

I.9. FINAL REMARKS

The purpose of this review was to detail a range of the most commonly used biomaterial hydrogels based on algae polysaccharide for transplantation of chondrocytes or stem cells in a cartilage regeneration strategy approach. The progress from the *in vitro* experimental cartilage TE research, using relevant cells types, to the *in vivo* scenarios was described throughout the demonstration of applicable results. Ultimately, some hydrogels have already been submitted through clinical trials, showing that it is possible to maximize the generated knowledge and attain the fabrication of medical products that make use of these systems.

With regard to their prevalence in the cartilage TE field, it is easily identified that alginate is the most common used polysaccharide followed by agarose, whereas polysaccharide κ-carrageenan and ulvan have only recently been proposed, reflected by the few found published reports. It is clear that these biopolymers provide a versatile class of hydrogels that may found widespread application in the field of regenerative medicine.

As seen throughout the studies detailed here, the type of hydrogel, concentration, cell intrinsic characteristics from a particular source or density and the cues incorporated play a large role in directing the fate of the final tissue engineered construct. Therefore, it is essential to determine and optimize the key regulator factors that influence cartilage regeneration.

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SECTION 2. DETAIL DESCRIPTION OF TESTING MATERIALS AND METHODOLOGIES APPLIED

Chapter II. MATERIALS AND METHODS

ABSTRACT

The aim of this chapter is to provide detailed description of all materials and experimental methods/techniques as well as the protocols used in this PhD thesis.
Whereas each chapter in this thesis contains the materials and methods used, herein it is intended to provide the reader with a more complete and comprehensive view of the detailed procedures and relevant information for the selection of the materials and cells, in addition to the physical and biological characterization assays, facilitating their use by others.

II.1. MATERIALS/POLYMERS

Hydrogels have acknowledged a widespread application in tissue engineering and regenerative medicine. They play important roles guiding the organization, growth, and differentiation of cells, granting physical support, as well as chemical and biological cues needed to form functional tissues [1]. There is a vast body of knowledge on hydrogels that have been proposed for research in tissue engineering applications however, the ideal support is yet to be found. The work described in the scope of this thesis focused on carrageenan, marine origin sulphated polysaccharides that exhibit GAG-like biological properties and which offer potentially safer compounds than mammalian polysaccharides, to be applied in cartilage tissue engineering strategies. Alginate and agarose were also used in different studies, described in chapter III and chapter VII, respectively.

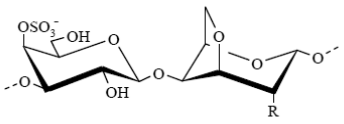
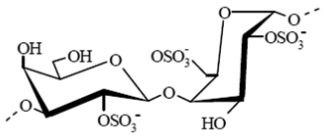
II.1.1. Carrageenan

Carrageenans are water-soluble sulfated polysaccharides widely utilized in food, pharmaceutical and cosmetic industries as they can form hydrogels at body temperature in the presence of gel-promoting salts. Gelation is still subject of debate and is affected by species of cations and concentration involving a coil-to-double helix conformational change, followed by aggregation of the ordered molecules in an infinite network [2]. These systems are novel and distinguish themselves from other hydrogels described in the literature by several phisico-chemical features. For example, carrageenan holds the ability of to become a gel at body temperature that heated turns into fluid, thus evidencing its thermoreversible characteristic. Carrageenans exists in three different forms, κappa (κ-) has one

sulphate group, while ιota (γ-) and lambda (λ-) correspond with the presence of two and three sulphate groups, respectively, per disaccharide unit (Table 1). Moreover carrageenans form *in situ* hydrogels under safe and mild conditions when potassium ions are added to the systems. They have a straightforward scale up gelation mechanism, thus leading to thydrogels with regular and reproducible shape, which can be processed into different formats, according to a specific target application. Another importer characteristic of carrageenan is the chemical composition resembling to the galactose or N-acetylgalactosamine containing glycosaminoglycans (GAGs); furthermore due the enclosed sulphate groups, they are similar to chondroitin sulfate, dermatan sulphate and keratan sulphate extracellular components [3].

Besides the phisico-chemical features, important biological advantages of carrageenan hydrogel over other encapsulating matrices are: the simple and readily manipulated system for encapsulated cells; a delivery substance and three dimensional hydrogel for tissue engineering cartilage; the ready dissolution of the hydrogel by agents that remove the potassium from the 3D hydrogel network formed.

Table II- 1. Structure of κappa (κ)-, ιota (ι)- and lambda (λ)- carrageenan. The disaccharides sequences of sulfate esters of κappa-, ιota-, and lambda-carrageenan are depicted, showing the β-1⟶4 and the α-1⟶3 bonds.

Types	κappa (κ)-carrageenan	ιota (ι)- carrageenan	lambda (λ) – carrageenan
Idealised chemical structure			
	->3)β-D-Galactose-4-sulfate (1->4) α-D-3,6-Anhydrogalactose (1->		->3)β-D-Galactose-2-sulfate (1->4) α-D-Galactose-2,6-disulfate (1->
	one sulfate group R = OH	two sulfate groups R = OSO_3^-	three sulfate groups

Iota and κappa -carrageenan used in this thesis were purchased from Sigma, Cat n° 22048 and Cat n° 22045, respectively, and used without further purification. The molecular weight of commercial extracts polymer ranges from 400 - 700 kDa [4, 5]. The manufacture of carrageenan involves hot extraction, clarification, evaporation, precipitation and drying and due to these processes, the obtained carrageenans are not pure as they show impurities from one another [6].

II.1.2. Alginate

Alginate, also known as alginic acid, is obtained from brown algae has been widely used in tissue engineering due to the characteristics like easy and mild hydrogel formation in the presence of divalent cations ensuring the preservation of cell viability and function. Besides its low toxicity, alginate is considered a slow biodegradable polymer and unable to interact with mammalian cells. The degradation mechanism is based on disintegration of the material due to the gradual exchange of gelling calcium ions with sodium [7]. Alginate is composed of (1-4)-linked β-D-mannuronic acid and α-L-guluronic acid units (Figure 1).

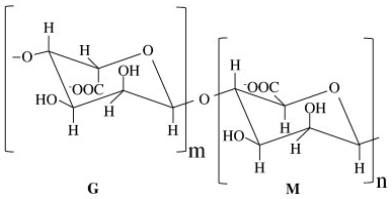


Figure II- 1. Chemical structure of alginate composed of guluronic acid (G) and mannuronic acid (M) units.

II.1.3. Agarose

Agarose is a natural based polysaccharide obtained from agar and has a neutral charge and gelation occurs at temperatures below 40 °C, whereas the melting temperature appears to be 90 °C. Agarose is a linear polymer consisting of alternating D-galactose and 3,6-anhydro-L-galactose units (Figure 2).

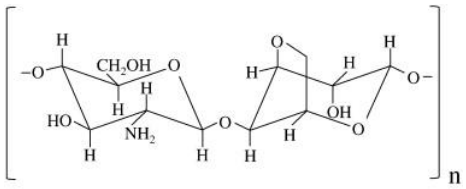


Figure II- 2. Chemical structure of agarose

Agarose has been widely studied for biomedical applications and thus it was used as a control material in chapter VII. Agarose powder was purchased from MP Biomedicals, Cat n° 800257

II.2. HYDROGELS DEVELOPMENT

For hydrogels development an aqueous solution was prepared by dissolving κ-carrageenan powder in distilled water at 60 °C, with constant stirring until complete and homogeneous dispersion [8].

Carrageenan solution has to be well dispersed to avoid the formation of lumps and thus to obtain a homogeneous hydrogel. Previous to use, all solutions were sterilized by steam power during 30 minutes at 121 °C. The solutions were prepared just before use, in order to avoid the initial thermal degradation steps which might affect the biomaterial response. Potassium chloride (KCl, P5405, Sigma) 5 % (w/v) solutions was used as reticulation agent. Upon cooling and after addition of the salt, an infinite variety of gels can be efficiently produced due to development of double helices of polymeric chains into the building blocks of a three-dimensional network. The selection of κ-carrageenan type for the majority of the studies performed under this thesis was based on prelimanary experiments performed, comparing κ-carrageenan with ιota carrageenan. Difference between the 2 types of carrageenan were found in terms of gelation, κ-carrageenan leading to stiff, brittle gels (with potassium salts) and the ιota type leading to elastic and gels (with calcium salts). Furthermore, in chapter III, alginate was blended with the 2 types of carrageenan, κappa and ιota, producing different results in terms of morphology, depending on concentration. Higher concentrations of the polymer, namely κappa type, could not be dropped easily due to the high viscosity, while lower concentrations of ιota type led to unstable gels. Regarding the cell studies, the cytotoxicity assays and the loading of chondrocytes revealed that κappa carrageenan type enabled higher cell viability than ιota type. Given that carrageenans classification is based on the sulphate content, with κappa having the lowest sulphate groups, the biological behavior seems to be dependent on the degree of sulfation, as such, for further experiments it was considered the κappa type.

II.2.1. Blended hydrogels based on carrageenan and alginate

In chapter III hydrogels blends of alginate with the two types of carrageenan were prepared and tested in the format of beads and fibers. The purpose of developing these blends was to combine specific properties of each polymer and obtain better results in terms of mechanical and biological characteristics.

II.2.1.1. *Preparation of bead from alginate and carrageenan mixtures:*

Carrageenan and alginate aqueous dispersions were prepared separately by dissolving each of the polymers in distilled water, heated up at 60 °C (for carrageenan) and 50 °C (for alginate) [9]. The tested polymers concentrations were from 2 until 3.5 % (w/v) and the ration of alginate-carrageenan blends were 5: 5; 7: 3 and 8: 2 (w/w). The hydrogel beads were obtained based on a droplet technique, which consisted in the extrusion of the liquid polymer from the tip of a syringe using a pump

(AL-1000, Alladin Programmable Syringe Pump) into the collecting bath of 100 mL KCl and $CaCl_2$ (Figure 3).

II.2.1.2. *Preparation of fibers from alginate and carrageenan mixtures:*

For the preparation of fibers the same steps, as described above, were followed to obtain the polymeric working solutions. The fibers were formed by the wet spinning technique, an easy and reproducible method, which consists in immersing the needle into the precipitation solution of $CaCl_2$*KCl. Each polysaccharide was ionically cross-linked with the corresponding cations, thus, alginate and ιota carrageenan prefer to form a stable gel with Ca^{2+} and κappa carrageenan with K^+. Several ratios of alginate to carrageenan blends were tested (blend of alginate with κappa carrageenan and blend of alginate with ιota carrageenan), namely 8:2, 7:3 and 5:5 with concentrations varying from 2 to 3.5 % (w/v) (Figure 3).

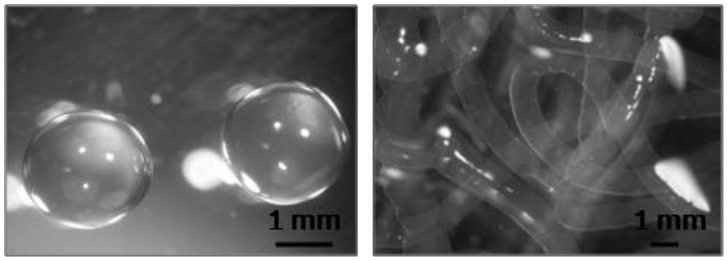


Figure II- 3. Optical microscopic images of the beads and fibres obtained from blending alginate and κ-carrageenan.

II.2.2. Carrageenan hydrogels – preparation of samples in the form of discs

In the work described in this thesis, it was optimized/used different techniques for producing carrageenan hydrogels samples in the form of discs. In chapters IV and V, the hydrogels were casted into cylinders and then sliced into discs while in chapters VI and VII, the hydrogels were molded into round thick membranes and then the disc samples were punched out, obtaining a more controllable thickness of the disc size. Since carrageenan is a thermosensitive polymer, just by allowing the polymeric solution to cool down for a few minutes at room temperature is enough to form a solid gel. In more detail, the first method consisted in casting the carrageenan solution into cylindrical moulds and once it gellifies, the cylinders were immersed in KCl for 15 minutes up to 30 minutes, in order to stabilize the 3-dimensional structure. Subsequently, the hydrogels cylinders were washed with phosphate buffered saline (PBS, P4417, Sigma) to remove the excess of KCl and cut, using a sterile

blade, into disc samples with dimensions of Ø 5 ± 0.01 x 2.5 ± 0.46 mm height (Figure 4). The other method to produce discs (chapter VI and VII) consisted in casting carrageenan polymeric solution into sterile plastic Petri dishes and allow cooling down at room temperature for 2-5 minutes (Figure 4). The hydrogel formation was further stabilized by cross linking with 5 % (w/v) potassium chloride and the discs were cut off from the thick membrane using a sterile punching device with various dimensions.

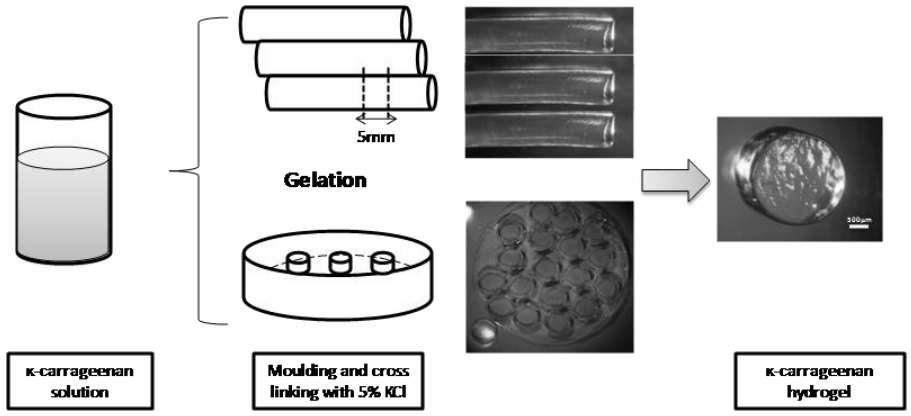


Figure II- 4. Schematic illustration of carrageenan hydrogel disc preparation

II.2.3. Agarose hydrogels

Agarose is a well known hydrogel that has been proved to be biologically compatible and thus it has been widely used for cell encapsulation in numerous *in vitro* and *in vivo* studies, envisioning TE applications [10-13]. For this reason, it was selected to be used in chapter VII as a control material. Agarose hydrogels discs were produced by dissolving 2% (w/v) powder prepared in sterile PBS and heated to 70 °C for 30 seconds, until complete homogenization was observed. The solution was then allowed to cool at room temperature, thereby decreasing the temperature and promoting gelation, afterwards punching off the discs samples.

II.3. CHARACTERIZATION OF THE DEVELOPED HYDROGELS

Physical properties provide important information about the processability of the 3D hydrogel also allowing predicting, to a certain extent, the biological outcomes. The physical analysis of the hydrogels developed in this thesis were analyzed by several techniques, including light microscopy, scanning electron microscopy, degradation/weight loss and swelling/water uptake assays or mechanical properties assessed by compression tests, as described below.

II.3.1. Optical microscopy

The size, shape and surface morphological characteristics of the developed hydrogels were examined using an inverted light microscope (Zeiss, Axiovert 40 CFL, 459306), and/or a stereomicroscope (Zeiss – Stemi 2000-C KL 1500 LCD, 459315) and images were obtained using a camera PowerShot G6, Canon. Freeze dried samples were produced by exposing the carrageenan hydrogels discs to lower temperature (-80 °C) and quickly transferring them to a freeze dryer (Telstar Cryodos-80, Telstar, Spain) where they were lyophilized during 2-3 days (Figure 5). This characterization was performed in the chapter III of this thesis.

II.3.2. Scanning electron microscopy (SEM)

The scanning electron microscope (SEM) was used to evaluate the morphology, pore shape, size and distribution providing images of the surface and cross sections of the hydrogel samples (Figure 5). In chapter III, prior to the SEM analyses (performed using a Leica Cambridge S360), the hydrogels were washed in PBS, fixed in a solution of 2.5% glutaraldehyde (in PBS), dehydrated in a gradient series of ethanol solutions and allowed to dry completely at room temperature before gold sputtered (Fisons Instruments, model SC502, England) for 2 minutes at 15 mA.

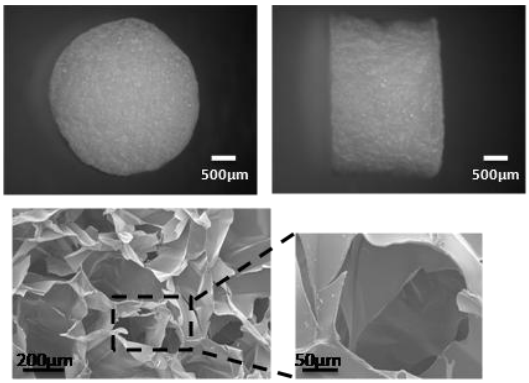


Figure II- 5. Macroscopic observation of the hydrogels obtained and SEM micrographs of the freeze dried hydrogels.

II.3.3. Degradation studies

Degradation behavior is an important parameter for assessing the suitability of hydrogels for a proposed biomedical application and was investigated in chapter III. The proposed hydrogel should

delivery the cells at the target site but also maintain its structure under physiological conditions providing a defensive role for the implanted cells for enough time to guarantee their functionality.

The degradation studies were carried out by immersion of the hydrogels discs (Ø 7 ±0.01 x 10±0.02 mm height, n=3) in either PBS, pH 7.4 or culturing medium (Dulbecco's Modified Eagle's medium, DMEM, supplemented with fetal bovine serum, FBS, Gibco, pH 7.4) at 37 °C. The hydrogels were lyophilized and weighed (initial weight) before being transferred to 15 ml Falcon tubes and soaked in 10 ml PBS or DMEM under constant agitation (60 rpm). The degradation solutions were changed every 7 days to restore the original level of ions activity. After each selected degradation time point, namely 1, 7, 14 and 21 days, the samples were washed thoroughly with PBS to remove traces of soluble degradation products, salts, or other impurities and then dried until constant weight was achieved; the dried samples were weighed (final weight) for determination of weight loss. The extent of degradation is commonly determined by calculating the percentage of weight loss (eq. 1):

$$Weight\ loss\ (\%) = \frac{Initial\ \ weig\,ht - Final\ \ weig\,ht}{Initial\ \ weig\,ht} \times 100 \qquad (1)$$

II.3.4. Swelling kinetics

The swelling is an intrinsic characteristic of hydrogels, as their networks are able to retain water in various percentages, dependent on their chemistry. In chapter IV of this thesis, the swelling behavior of the developed hydrogels was studied in a PBS solution (different pH values were tested) and in culture medium. For this purpose, a weighed amount of each hydrogel formulation was immersed in each testing solution (PBS, DMEM = pH 7.4) and incubated at 37 °C under static conditions. The influence of the pH of the PBS solution on the water uptake of κ-carrageenan hydrogel was also assessed. The swelling ratio was calculated using equation 2.

$$\%\ Equilibrium\ swelling\ ratio\ (\%\ ESR) = \frac{W_s - W_d}{W_d} \times 100 \qquad (2)$$

W_s: weight of swollen gel after reaching equilibrium value under specified environmental conditions (PBS or DMEM); W_d: weight of the dried gel.

To measure W_s, the swollen hydrogels were removed from the PBS or DMEM and immediately weighed with a microbalance after the excess of water lying on the surfaces was absorbed with a filter paper. Three samples were used for each testing condition (n=3).

II.3.5. Static compression tests

In chapter III, uniaxial compression tests were performed to characterize the mechanical behavior of the produced 2.5 % (w/v) alginate-carrageenan hydrogels discs (Ø 15±0.51 mm x 5.5±0.46 mm height). The tests were carried out at room temperature using an universal mechanical testing machine (Instron 4505 Universal Machine). Mechanical testing was performed under compression using a crosshead speed of 5 mm/min and the results averaged from tests conducted in at least 10 specimens [14].

II.4. IN VITRO CELL CULTURE STUDIES

In vitro cell culture studies performed under the scope of this thesis include experiments/assays designed to evaluate the *in vitro* biocompatibility of the developed hydrogels, namely cytotoxicity and immune response evaluation assays, and studies concerning the encapsulation of different cells into the developed systems.

II.4.1. Cell used in the different experiments

In this section it is described the type of cells and their isolation/culturing methodologies, that were used in the biocompatibility studies and in the *in vitro* studies designed to assess the behavior of cells encapsulated in the developed hydrogels.

II.4.1.1. *MOUSE FIBROBLAST-LIKE CELL LINE (L929 CELLS)*

For the cytotoxicity assessment of the materials described in chapter III, IV and VII, a mouse fibroblast-like cell line, L929 (ECACC- European Collection of Cell Cultures, UK) was used. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM, D5523, Sigma), supplemented with 10 % fetal bovine serum (FBS, 10270-106, Invitrogen) and 1% antibiotic/antimycotic (A/B, 15240-062, Invitrogen) solution. The cell density of L929 cells used for the cytotoxicity tests was $2x10^4$ cells/ mL, which was seeded in 96-well cell culture plates ($4x10^3$ cells/well plate, 200 µl/well plate), in order to reach 70 % confluence for 24 h of incubation at 37 °C, in a humidified atmosphere with 5% CO_2.

II.4.1.2. *HUMAN POLYMORPHONUCLEAR NEUTROPHILS CELLS, (HPMNS)*

For the *in vitro* immunological response assay, performed in chapter VII, it was used human polymorphonuclear neutrophils cells (PMNs) isolated from heparinized peripheral blood collected from healthy volunteers (under a protocol previously established with the Portuguese Blood Institute – *Instituto Português do Sangue* - IPS), following protocols previously established [15].

The biological response following implantation of a material in the body such as inflammation takes place triggering circulatory inflammatory cells to the injury site, first PMNs. Within 24 hours, macrophages and in two or three days, lymphocytes begin to migrate to the site of injury, later other inflammatory cells, such as mast cells and eosinophils will coordinate the continuing of the inflammatory response. PMNs are crucial in development of an inflammatory response dictating the progression of the host immune system reaction by their capacity to produce cytokines, such as interleukins, acting as phagocytes of foreign bodies and leading to the production of oxygen radicals, Reactive Oxygen Species (ROS) as a defense mechanism [16].

The heparinized blood was placed in 6 % Dextran (D8906, Sigma) with PBS without Ca^{2+} and Mg^{2+} for 20 min, afterwards Histopaque® -1077 (1077, Sigma) was carefully added (Figure 6). After 25 min, the red pellet cells was resuspended with PBS without Ca^{2+} and Mg^{2+} and centrifuged for 25 min at 21 °C and 2400 rpm. In order to lyse the erythrocytes from the cell pellet, distilled water was added and several centrifugations were performed, before counting the cells. The hPMNs cells were kept at 4 °C until further use, within a maximum of 2 h.

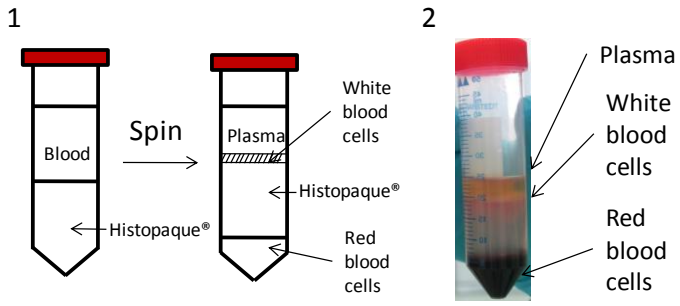


Figure II- 6. Schematic representation of the protocol used for the isolation of human polymorphonuclear neutrophils cells, hPMNs.

II.4.2. Cells used for encapsulation experiments

In the work described in chapters III, IV and V, cryopreserved mouse derived cells (ATDC5 cell line), human nasal chondrocytes (hNCs) and human adipose derived stem cells (hASCs) were used after thawing/expansion procedures, while in chapter VII, freshly isolated hASCs were expanded to use in the described encapsulation experiments. The selection of a cell source to promote efficient regeneration is a major issue to be considered in the design of a therapeutic strategy. Ideally, a cell source should allow harvesting in large amounts with minimal morbidity, enable high number of cells, be easy to maintain/expand *in vitro*, and to guarantee phenotypic stability and functional suitability, have no issues of immunogenicity or disease transmission risks and be of low cost. In this context it

was motivating to work with different cell sources and to assess their behavior in terms of proliferation and chondrogenic potential envisioning applications in cartilage repair.

II.4.2.1. *ATDC5 CELL LINE*

ATDC5 is a cell line established from chondrocytic cells of a mouse embryonal carcinoma (mouse 129 teratocarcinoma AT805 derived, ECACC, UK). ATDC5 cells were selected to use in chapter III and V since they are biologically active in a 3D environment and have been extensively studied to assess their role in producing, maintaining, and remodeling the cartilage ECM [17]. Cryopreserved ATDC5 were thawed by placing the frozen cell vial in a 37 °C water bath. When the vial was partially thawed, the cells were removed from the bath and centrifuged with basic culture medium to remove the cryoprotectant (dimethyl sulfoxide, DMSO, 156914). The cell pellet was resuspended in culture medium, cultured in flasks, and allowed to attach for 24 hours prior to medium exchange. The cells were expanded in culture medium composed of Ham's F-12 medium (D-MEM/F-12 (1:1), 42400-028 Gibco) supplemented with 10% FBS (Gibco), 2 mM L-glutamine (G854, Sigma), and 1% antibiotic solution, until obtaining the necessary number of cells for the experiments the encapsulation experiments.

II.4.2.2. *HUMAN NASAL CHONDROCYTES (HNCS)*

Nasal cartilage was harvested from the nasal septum of adult patients undergoing reconstructive surgery under a protocol established with Hospital de S. Marcos, Braga, Portugal. For the isolation of the hNCs, the human nasal septum cartilage tissue samples were cut into pieces, washed in a sterile PBS solution and incubated in 20 mL of trypsin-EDTA solution for 30 min at 37 °C with agitation. Then, trypsin was removed and 20 mL of filter sterilized collagenase type II solution (2 mg/mL) in basic medium was added, and the mixture incubated for 12 h [18]. Subsequently, the digested tissue and cell suspension solution was centrifuged at 200×g for 7 min and the supernatant removed. Finally, the cell pellet was washed with lysis buffer, centrifuged several times and resuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium - high glucose (DMEM, 5671, Sigma), containing 10 mM HEPES (H403475, Sigma) buffer pH 7.4, 1% antibiotic-antimycotic, 20 mM L-alanyl glutamine (G8541, Sigma), 1x MEM non-essential amino acids (MEM NEAA, 11140-035, Sigma) and 10% (v/v) FBS supplemented with 10 ng/mL basic fibroblast growth factor (bFGF, AF100-18B, PeproTech). The expanded nasal chondrocytes were used in the work described on chapter V.

II.4.2.3. *ADIPOSE DERIVED STEM CELLS (hASCs)*

Adipose-derived mesenchymal cells (hASC) are an attractive type of adult stem cells, since it is possible to obtain large amounts of their source tissue with low donor site morbidity and have the ability to commit to a range of different lineages [19]. The chondrogenic differentiation capacity of hASCs once encapsulated in carrageenan hydrogel was assessed in chapters IV and V. Furthermore, in chapter VI the chondrogenic differentiation potential of hASCs was evaluate after cryopreservation of the cell-hydrogels constructs.

Adipose tissue samples were obtain under a previously established protocol with the Department of Plastic Surgery of the Hospital da Prelada, Porto and approved by the local Ethical Committee. The isolation protocol implies enzymatic digestion of the tissue samples with collagenase type II (C6885, Sigma) in Dulbecco's Phosphate Buffered Saline (DPBS, 21600-044, Invitrogen) for 45 min at 37 °C under gentle stirring. Afterwards, filtration was performed using a 100 μm filter mesh (Sigma) and after several centrifugations, the stromal vascular fraction (SVF) was washed with lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH= 7.3) to remove the red blood cells [20]. The SVF was plated and 2-3 days after, the remaining non-adherent cells were removed with repeated PBS washings. The adherent hASCs were cultured with media changes on every three days [21, 22]. hASCs were expanded in culture medium composed of alpha Minimum Essential Medium (α-MEM, 12000-063 Gibco, Invitrogen) with 10 % FBS (10270-106 Gibco, Invitrogen; heat inactivated), 1 % Antibiotic-Antimycotic (15240-062, Invitrogen) and sodium bicarbonate (S5761-NaHCO3, Sigma). Human ASCs were subcultured at a density of 3.5 x 10^3 cells/cm^2 and incubated at 37 °C in a humidified atmosphere of 5 % CO_2. Confluent cultures were passaged with 0.05 % trypsin (25300-062 Invitrogen). The enzymatic treatment was quenched in the presence of FBS and cell counts were done using a hemocytometer. Only passage 3 (P3) hASCs were used in the experiments. The hASCs used in chapter IV and V, were isolated from lipoaspirates, while the hASCs from chapter VI were obtain from compact tissue samples (Figure 7), both from subcutaneous sites. For the cell culture experiments in chapter VI, the adipose tissue derived stem cells were from four female donors with a mean age of 35.25 years (± 8.55) and BMI (body mass index) of 26.26 (± 2.74). The phenotype of human adipose stem cells has been extensively investigated in previous studies using tissue sample from same anatomical site (subcutaneous), harvested with similar technique and isolation following similar procedures (digestion with collagenase) by [23, 24] including by our research group [25-27].

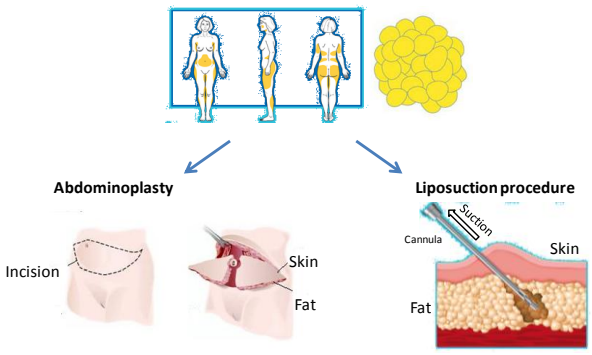


Figure II- 7. Harvesting sites and procedures to obtain the adipose tissue.

II.4.3. Cytotoxicity screening

In order to establish the possible toxic effects of leachable eventually released from the developed hydrogels, indirect cytotoxicity tests were performed, using extracts of the materials, following methodologies based on ISO/EN 10993 part 5 guidelines [28, 29]. Cytotoxicity screenings were accomplished in chapter III, IV and VII of this thesis (Figure 8).

II.4.3.1. *MEM EXTRACTION TEST*

The developed hydrogels were extracted in complete culture medium for 24 h at constant temperature (37 °C) and agitation (60 rpm) [30]. The ratio of material to extract fluid was constant and equal to 3 cm^2/mL. To assess the short-term cytotoxicity of the developed hydrogels, latex rubber was used as positive control for cell death due to its high cytotoxicity to cells, and culture medium was used as a negative control representing the ideal situation for cell growth. Extracts of the different polymers were filtered (filter pore size: 0.22 μm) and placed in contact with the monolayer of the L929 cell line. For this purpose, the culture medium from the well-plates with the L929 cells monolayers was removed and an identical volume (200 μl/well) of extraction fluid of study materials or rubber latex extracts (positive control) and negative controls (standard culture medium) was added. The MTS assay was performed to assess the possible cytotoxicity of hydrogels extracts determining the metabolic activity of the cells.

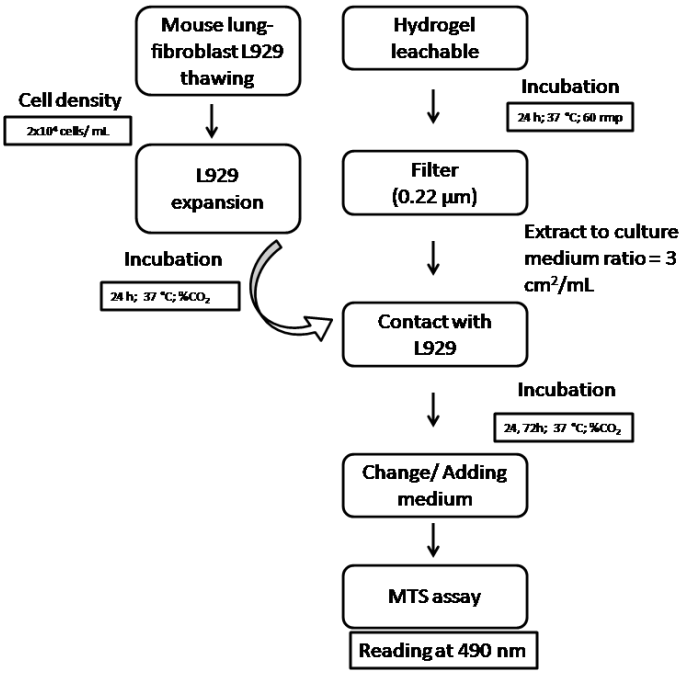


Figure II- 8. Schematic representation of the cytotoxicity assay

II.4.3.2. *METABOLIC ACTIVITY*

The metabolic activity of cells in contact with extracts of the developed hydrogels was evaluated by the MTS assay (Cell Titer 96® Aqueous Solution Cell Proliferation Assay, Promega), in chapter III, IV and VII. The absorbance of the resulting solution in each well was recorded at 490 nm using an automated Multi-Mode Microplate Reader (Synergy™ HT, Bio-Tek Instruments). The results presented in chapter III, were as percentage of cell viability as hydrogel O.D (optical density) was normalized to the negative control using the following equation:

$$Cell\ viability\ (\%) = \frac{OD\ sample}{OD\ control} \times 100 \qquad (4),$$

where OD sample is the optical density obtained in the cells exposed to each extract and OD control is the OD obtained in the cells incubated with the culture medium only (negative control). Furthermore, in chapters IV and VII the MTS results after cytotoxicity tests were showed as OD values of hydrogel material, TCPs, the cell component (negative control) and latex (positive control).

II.4.4. Chemiluminescence - Reactive oxygen species quantification assay

In vitro culture models that estimate reactive oxygen species (ROS) levels within cells and/or their release into the culture media provide a valuable tool in understanding the mechanisms that lead to

cell loss and will further explain *in vivo* inflammatory response. The presence of superoxide anions and hydroxyl radicals in cell culture with the tested hydrogels was detected by a chemiluminescence-based method. The isolated hPMNs, were used at a final concentration of 1.3×106 cells/mL. For the detection of reactive oxygen species, to each 100 µL of cells suspension with the two cell stimulants, namely 8 µg/mL PMA/PBS (phorbol 12-myristate 13-acetate, P8139, Sigma) and 10 µg/mL fMLP/PBS (formyl-methionyl-leucyl-phenylalanine, F3506, Sigma), was added 100 µL of 1.5 mM luminol/PBS (09253, Sigma) or 100 µL 5.4×10^{-5}M lucigenin/PBS (B49203, Sigma) [16]. Control conditions were simultaneously set using no cell stimulants. Samples with or without materials were kept on ice until asses. The chemiluminescence was read with a microplate reader (Sinergy HT, Bio-TeK Instruments) and the results were obtained in terms of total number of counts per time period.

II.4.5. Cell encapsulation experiments

Cell encapsulation studies were performed to assess the cellular response of the developed hydrogels and to further select/optimize the formulation as well as the encapsulation conditions. The temperature-induced gelation of carrageenan permits easy formation of hydrogels under mild conditions, enabling its application as an *in vitro* cell-carrier or as an *in vivo* injectable system (Figure 9). Encapsulation studies were performed in chapter III, IV, V and VI.

II.4.5.1. *Encapsulation of ATDC5 cells into alginate/carrageenan hydrogels*

In chapter III and V, ATDC5 cells were encapsulated into alginate/carrageenan hydrogels. After trypsinization and centrifugation, the cell suspension obtained was diluted in order to encapsulate $1x10^6$ cell/ml of the polymeric solution to produce the beads/fibers as previously described. The beads/fibers with encapsulated cells were then placed into non-adherent 24 well-plates and maintained in culture for several periods of time (1, 7, 14 and 21 days) at 37 °C in a CO_2 incubator. At the end of each time of culture, the beads/fibers with the encapsulated cells were retrieved, washed with PBS solution and maintained at 4 °C in PBS for further characterization studies.

II.4.5.2. *Encapsulation of hASCs in κ-carrageenan hydrogels*

In chapters IV, V and VI, it was performed the encapsulation of hASCs cells in κ-carrageenan hydrogels. The κ-carrageenan aqueous dispersions were prepared just before being used and sterilized as described in previous sections. hASCs were detached by trypsin and centrifuged at 200×g for 7 min.

Cells were resuspended in sterile PBS solution, counted using a haemocytometer and finally centrifuged. The supernatant was discarded and κ-carrageenan 1.5 % (w/v) solution was added to the cells to obtain a final concentration of 5×10^6 cells·mL^{-1}. The carrageenan aqueous solution and the cells suspension was mixed until complete homogenization (Figure 9). Hydrogel discs loaded with cell were prepared using sterile polystyrene cylinder moulds and allowed to rest until forming a solid gel. The chondrogenic induction medium was composed of Dulbecco's Modified Eagle's Medium-low glucose (D5523-DMEM Sigma, USA), supplemented with 1% Antibiotic-Antimycotic, ITS+1 Liquid Media Supplement (I2521- insulin-transferrin-selenium - liquid media supplement, Sigma, USA), 17mM L-ascorbic acid (A4544 Sigma, USA), 0.1M sodium pyruvate (P4562 Sigma, USA), 35mM L-proline (P5607 Sigma, USA), 1mM dexamethasone and 10ng/ml of human TGF-β1(Transforming Growth Factor-β1, 14-8348, eBioscience). Additional controls consisted of κ-carrageenan hydrogel samples without cells, were kept in the same culturing conditions for the same selected time periods. At the end of the pre-established culture periods (21 days), the samples were retrieved, washed with PBS solution and further processed according to the characterization assays to be performed, as described below.

For the cryopreservation study performed in chapter VI, the hASCs encapsulated at a density of 5×10^6 cells/cm^3 into the carrageenan hydrogels were cultured for 14 days in either chondrogenic or basal medium. Two-weeks time point was selected based on previous studies on hASCs chondrogenic differentiation [31] that suggested that this is the period of time necessary in setting chondrogenesis with ECM production [32]. The overall time-frame of the encapsulation studies, namely 21 days of culture was selected with basis on previous works with similar aims, using encapsulated stem cell in hydrogel for chondrogenic differentiation [33-35]. Though it will be interesting to verify the outcomes in a long time experiment, envisioning the clinical application of the proposed strategy, we were mostly concerned with the ability of the hydrogels to promise early pre-differentiation as a long term *in vitro* culturing, before implantation, is not interesting in a clinical/industrial scenario.

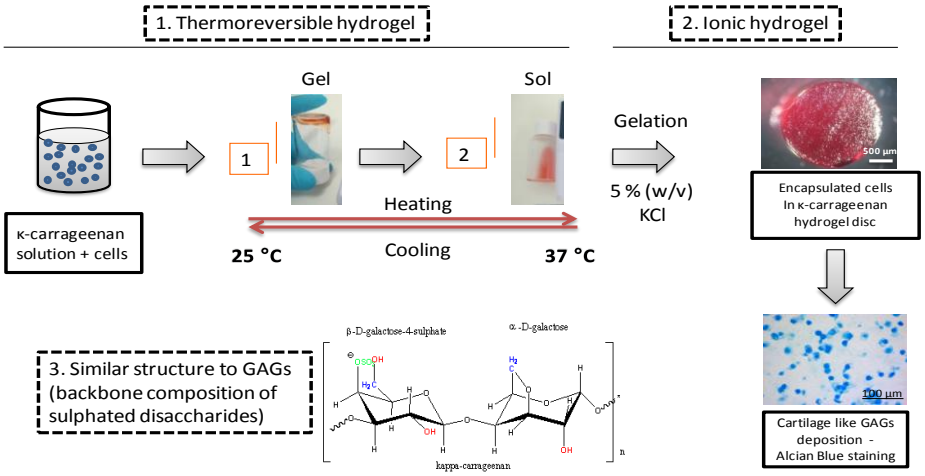


Figure II- 9. Schematic representation of carrageenan hydrogel preparation.

II.4.6. Cryopreservation studies

One of the main prospects of cartilage tissue engineering is the possibility of developing custom-made regenerative medicine solutions on an individual patient basis. The establishment of efficient preservation and storage procedures will provide products prepared in advance, scaled up, assuring immediate availability and accessibility for the clinical use which could be adapted to an autologous immediate solution, leading to "out of shelf" tissue substitutes. With this in mind, in this thesis it was investigated the influence of standard cryopreservation protocols on the chondrogenic differentiation characteristics of human adipose derived stem cells encapsulated in κ-carrageenan hydrogels, as a promising tissue engineering product to be applied in cartilage regeneration (chapter VI). The cryopreservation process was based on the following protocol: each sample was individually placed in a polypropylene cryovial (479-0821, VWR) containing a solution of 10 % (v/v) dimethyl sulphoxide (CryoSure DMSO, 11-32-30216, Wak-Chemie Medical GMBH) in FBS. The samples were cooled down to 4 °C using an ice bath (30 minutes). Afterwards, cryovials were moved into a conventional freezer (-20 °C; 1-2h) and then moved into a -80 °C freezer. After 12 hours at -80 °C, samples were stored inside a liquid nitrogen tank (Statebourne Biosystem) at -196 °C for one month.

II.5. CHARACTERIZATION OF CELL-LADEN HYDROGELS SYSTEMS

Cell-laden carrageenan hydrogel systems were characterized in terms of cellular viability and proliferation as well as of chondrogenic differentiation features. Furthermore, morphological characteristics and the production of extracellular matrix components typical to cartilage were also considered and are described in the following sections of this chapter.

II.5.1. Morphological cell evaluation and distribution

Cell morphology and distribution throughout a 3D matrix was evaluated using optical microscopy techniques in chapters III and VI. The full structure and morphological characteristics of the developed discs with encapsulated cells was observed under inverted light microscope (Zeiss, Axiovert 40 PG-HITEC) equipped with a digital camera (Axiocam MRc5, Zeiss).

II.5.2. Water content and degradation rate

The environment in which cells are grown influences the cellular response, affecting cell viability and proliferation, therefore the water content/ degradation rate of the developed hydrogels was evaluated in simulated physiological conditions (chapter V). Hydrogel samples (111.15 mg ± 12.66) prepared according to the procedure described above and accurately weighed (w_s) were incubated for 7, 14 and 21 days in fresh culture medium and PBS buffer. Simultaneously, samples of hydrogels loaded with hASCs, hNCs and ATDC5 (prepared as described in the section above) were immersed in specific culture medium for each type of cell and incubated at 37 ^{0}C for the same time periods. At the pre-determined time intervals, the medium/PBS was removed from the samples, the hydrogels were lyophilized and the dry weights were measured (w_d). The water content of hydrogels was calculated from the equation $(w_s - w_d)/w_s \times 100$. The degradation rate was defined as the time needed for the gel to degrade and for calculation we considered the equation 5. The medium was replaced twice a week and the studies were performed in triplicates.

$$Degradation\ rate\ (\%) = \frac{Final\ \ weig\,ht}{Initial\ \ weig\,ht} \times 100 \qquad (5)$$

II.5.3. Dynamic Mechanical Analysis (DMA)

Dynamic mechanical analysis is a technique used to study and characterize the viscoelastic nature of hydrogels, as a response to stress, temperature and frequency, and determines their time-dependent mechanical performance. Cells and tissues exhibit a viscoelastic behavior that an implantable hydrogel should match in order to be successfully applied in a tissue engineering strategy [36, 37]. In chapters IV and VI, dynamic mechanical analysis (DMA) was conducted to analyze the stiffness of carrageenan hydrogel discs with or without laden hASCs cells cultured at different time points exposed or not to chondrogenic differentiation supplements. The carrageenan hydrogel concentration, and the disc sample size used in this study, as well as the culturing time assessed are summarized in table II.2.

Table II- 2. Samples/conditions used in DMA analysis.

DMA ANALYSIS	HYDROGEL CONCENTRATION	DISC SIZE	TIME OF CULTURE
CHAPTER IV	2 % (w/v)	8 ± 0.01 mm ∅ x 2.5 ± 0.38 mm height.	21 days of culture
CHAPTER VI	1.5 % (w/v)	5 mm ∅ x 3 mm height.	before and after cryopreservation

The carrageenan hydrogel discs prepared (as described in the hydrogel development section), were subjected to compression cycles of increasing frequencies ranging from 0.1–10 Hz with constant strain amplitude (50 μm) using DMA equipment (TRITEC Admin 8000B, Triton Technology). A small preload was applied to each sample to ensure that the entire disc surface was in contact with the compression plates before testing and the distance between plates was equal for all samples being tested. The mechanical analysis results were presented in terms of two main parameters: storage modulus (E′ - the in-phase, elastic component) and loss modulus (E″ - the out-of-phase, viscous component). The values for the compression modulus were collected along the days of culture and damping factor was presented as well.

II.5.4. Cell viability

The viability of encapsulated cells was evaluated by labeling the cells with fluorescent dyes like Calcein AM, propidium iodide and by using the colorimetric MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium).

II.5.4.1. *CELLULAR VIABILITY BY CALCEIN AM – FLUORESCENT DYE*

The fluorescent calcein AM is a cell-permeant and non-fluorescent compound which is hydrolyzed in live cells being the fluorescence intensity proportional to the amount of live cells. Single fluorescence labeling, with calcein AM only, was carried out in chapter III and IV, to assess the viability of ATDC5 and hASCs cells encapsulated in hydrogels. For this assay, the samples were washed with PBS, placed in a new well plate with 1 mL of DMEM and 2 μL of a Calcein-AM solution (at 1/1000 dilution) and incubated for 15-30 min at 37 ^{0}C in 5 % CO_2. Afterwards the hydrogels were rinsed twice in PBS and observed under a reflected/transmitted light microscope (Zeiss, Axiocam MRc5).

Live/dead assay with propidium iodide (staining for dead cells) was performed in chapter VI to assess the viability of hASCs cells encapsulated in κ-carrageenan hydrogels. In this case, samples were collected, placed in a 48-well plate, and washed with PBS. Subsequently, 500 μL of 1 mg/mL Calcein AM in PBS (C3099, Invitrogen) was added to each sample for 10 min at 37 °C and later washed with sterile PBS. Afterwards, the samples were incubated with 300 μL of 1.5 mM propidium iodide in PBS (PI; 1:1000, P1304MP, Invitrogen) for 5 min at room temperature, previous to microscopic analysis (Reflected/Transmitted light Microscope, Axioimager Z1M, Zeiss).

II.5.4.2. *METABOLIC ACTIVITY*

This MTS test (described previously in section 4.3 – cytotoxicity screening) was also used to evaluate the metabolic activity of cells encapsulated in the developed hydrogels, in chapter III and V at different time periods, specifically 1, 2 and 3 weeks. In this case, the cell–hydrogel constructs were washed in PBS and placed in a mixture containing serum-free cell culture medium DMEM (without phenol red) and MTS reagent at a 5:1 ratio. Incubation for 3h at 37 °C in a humidified atmosphere containing 5% CO_2 followed, at the end of which 100 mL was transferred to 96 well plates and the OD determined at 490 nm. Controls consisting of hydrogels without cells were also used. Thus, the alginate-carrageenan polymers with encapsulated ATDC5 cells were assessed for their metabolic activity in chapter III. The MTS compound bioreduction by metabolically active cells such as hASCs, hNCs and ATDC5 cells, into a brown formazan product by dehydrogenase enzymes was assessed in chapter V as well. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living viable cells in culture.

II.5.5. Cellular content assay (dsDNA quantification)

The proliferation of the encapsulated cells, ATDC5, hNCs, hASCs in the κ-carrageenan hydrogels, was determined using a fluorimetric double-strand DNA quantification kit (Quant-iT™ PicoGreen® dsDNA reagent, Molecular Probes, Invitrogen). The cell proliferation assay used in this thesis (chapters III, IV, V and VI) is a sensitive fluorescent nucleic acid staining, measuring the fluorescence produced when PicoGreen dye (Molecular Probes, P-7589) is excited by UV light and bounds to double-stranded DNA (dsDNA) present in the tested sample.

For this purpose, samples collected after culturing time were transferred into 1.5 mL microtubes containing 1mL of ultrapure water. The cell-hydrogel constructs and the hydrogel alone (without cells), used as control, were incubated for 1 h at 37 °C in a water bath and were then stored in a -80 °C

freezer until they were tested. Prior to dsDNA quantification, the cell-hydrogels were thawed and sonicated for 15 min. Samples and standards (ranging from 0 to 2 μg· mL^{-1}) were prepared and mixed with a PicoGreen solution in a 200:1 ratio and were placed on opaque 96-well plate. The procedure followed was according to the manufacturer's instructions [38]. The plate was incubated for 10 min in the dark, and fluorescence was measured on a microplate reader (Synergy™ HT, BioTek Instruments) with an excitation of 485/20 nm and an emission of 528/20 nm. A standard curve was created, and sample DNA values were read from the standard graph.

II.5.6. Histological characterization

The histocytological techniques include preservation, fixation, sectioning and staining technique to allow for visualization using a microscope (source: MeSH - Medical Subject Headings). In chapters III, IV, V and VI samples of cell-laden hydrogels retrieved at different culturing time-points were processed for histocytological analysis. The samples were fixed with 3.7 % formalin, processed to remove water from tissues (spin tissue processor, Microm STP120 Inopat) and replace it with an embedding medium (embedding center, Microm EC350-1/EC350-2 Inopat) to allow micro sections (3-4 μm) to be cut off (microtome, Microm HM355S Inopat). Since most staining are aqueous solutions, deparaffinization is a common step prior staining described below. After all staining performed, discussed in next section, all slides were dehydrated through series of alcohol immersions from 30 % until 100 % alcohol. Then, sections were allowed to air dry overnight. The final step for all the staining performed was the immersion in the clearing agent Histoclear® (National Diagnostics) or xylene (1.08681.1000, VWR) for 1-2 minutes and mounted using resinous medium, Microscopy Entellan® (Merck &Co., Inc.) for later observation. Stained sections were observed under a light microscope (Reflected/Transmitted light Microscope, Zeiss).

II.5.6.1. *H&E*

Hematoxylin and eosin (H&E) staining was performed to observe general cell morphology and overall cell/matrix distribution once in cell laden carrageenan hydrogel. Hematoxylin, a basic dye, stains nuclei blue due to an affinity to nucleic acids in the cell nucleus; eosin, an acidic dye, stains the cytoplasm pink. Other tissues such as connective tissue or decalcified bone matrix also stain pink with eosin while mucus and cartilage will stain a light blue color [39].

H&E staining was conducted in automatic staining equipment (Microm HMS 740) in chapters III, IV and VI. After hydration, sample sections were coloured with Papanicolaou Harris hematoxylin (05-

12011/L, Bio-optica) for 3 minutes, washed in running tap water, and afterwards a blue stain enhancement was performed by an immersion in 0.5 % ammonium hydroxide solution (05002, Sigma) for 5–10 seconds. The sections were washed in running tap water and stained in Eosin-Y (05-M10003, Bio-optica) for 30 seconds. Finally, slides were dehydrated through series of alcohol immersions from 30 until 100 % (v/v) alcohol. The stained cells were visualized under a light microscope (Reflected/Transmitted light Microscope, Zeiss Germany) and images were taken by a digital camera (Axion MRc5, Zeiss).

II.5.7. Typical proteoglycans staining

Alcian blue (A3157, Sigma), Safranin O (84120, Sigma), Toluidine blue (T3260, Sigma) were used to evaluate cartilage ECM components deposition, namely glycosaminoglycans on samples that were collected at the end of the experiment.

II.5.7.1. *Alcian blue staining*

Alcian blue [40] is a cationic dye, that stains acid mucosubstances and acid glycosaminoglycans, which may be present in cartilaginous tissues. Alcian blue staining was performed by rinsing the sections or the cells monolayer in 3 % acetic acid (151785, Sigma) and incubating them in 1 % Alcian blue solution from 30 minutes to 18 hours. After that, the stain was poured off, and sections were counterstained with aqueous neutral red (861251, Sigma) for 1 min and dehydrated. The sections were then rinsed with distilled water and observed at the microscope. This staining was performed in chapters IV and V.

II.5.7.2. *Safranin O staining*

Safranin O is a cationic dye frequently used as a counterstain and for the detection of mucin, cartilage [41] and mast cells. The Safranin O staining consisted of staining the sections with Weigert's iron hematoxylin working solution for 7 minutes, fast green (FCF, 44715, Sigma) for 5 minutes and 0.1 % safranin O for 5 minutes. Sections were washed after each staining step, left to air dry and then mounted for visualization. This staining was performed in chapters IV and V.

II.5.7.3. *Toluidine blue staining*

Beside its many roles as heparin antagonist and an antibacterial agent, Toluidine blue [41] is a basic nuclear and metachromatic dye that stains mucopolysaccharides. Toluidine blue solution was prepared

by dissolving 1 % of toluidine blue in distilled water containing 0.5 g of sodium borate (Riedel-de-Haën), followed by filtering and the sections were dipped in for 2–3 s. Afterwards, the stain was poured off and the samples washed, dehydrated, cleared in xylene and mounted for further analysis. This staining was performed in chapters IV and VI.

II.5.8. Immunohistochemistry analysis for collagen type II and type I

Immunodetection of specific cartilage proteins is a very precise technique in which specific antibodies are used to detect proteins or other molecules present in a given sample. Sections of samples corresponding to all experimental conditions namely, κ-carrageenan hydrogels loaded with stem cells exposed or not to chondrogenic conditioned medium (described in chapters IV and VI) were obtained as described in the section above. Immunocytochemistry was performed according to R.T.U. Vectastain Universal Elite ABC kit (PK 7200) kit from VectorLabs, using R.T.U Normal Horse Serum to avoid unspecific reactions, a biotinilated secondary antibody (R.T.U. Biotinylated Universal Antibody) and a Peroxidase Substract Kit (Vector SK-4100), 3,3'-Diaminobenzidine (DAB) was the colorimetric substrate used. In detail, before removing the paraffin, the slides were warmed, and the antigen retrieval was performed for 20 minutes at 95 °C using 10 mM citrate buffer. Sections were washed in PBS 10 to 15 minutes, and endogenous peroxidase activity was quenched with hydrogen peroxide (31642, Sigma) in 50 % methanol/tap water for 5 min. Afterwards, samples were washed with PBS and blocked with 2.5% horse serum from R.T.U. Vectastain® Universal Elite ABC Kit (PK-7200, Vector Laboratories) for 1 h to avoid nonspecific staining. Sections were further incubated with primary antibodies (collagen type II and collagen type I; mouse antitype II collagen - MAB1330 and mouse antitype I collagen - MAB3391, Chemicon or rabbit anti-collagen I antibody - ab292, Abcam) overnight at 4 °C, in a humidified atmosphere. Then slides were washed with PBS for 10 min each and incubated with secondary antibody (R.T.U. Vectastain® Universal Elite ABC Kit) for 1 h at room temperature, again in a humidified atmosphere. The remaining protocol was performed according to that described in the in the DAB substrate kit for peroxidase (SK-4100, Vector Laboratories). Slides were washed in water for 5 min and then counterstained with haematoxylin for nuclei visualization and finally, slides were mounted. Controls were performed using normal horse serum replacing the primary antibodies. The samples were visualized under a light microscope and images obtained using a camera (Axion MRc5, Zeiss).

II.5.9. RNA isolation and Gene expression analysis (RT-PCR)

The experimental work within chapters IV, V, and VI involved the use of molecular approaches to detect cartilage specific markers namely *Sox9*, *Aggrecan*, *Collagen X*, *Collagen type I*, *Collagen type II,* on cell laden hydrogels upon different periods of culturing and/or different culturing conditions. Real-time PCR is semiquantitative/quantitative method that measures in real time the quantity of a given codifying molecule in a sample, enabling to determine their regulatory profile, that can be up or downregulated, or alternatively exhibit no significant variation.

For this purpose, total RNA was extracted from cell-hydrogel constructs using TRI Reagent® RNA Isolation Reagent (T9424, Sigma) according to the provided technical data sheet. Samples of each condition were collected at defined time periods, washed twice with PBS, added in TRI Reagent® (800 μL) and vigorously mixed during 10 s and stored at -80 °C until the analysis was performed. At this point, 160 μL of chloroform was added, incubated on ice for 15 min and centrifuged at 13.000 rpm for 15 min at 4 °C to establish a three-phase composition in the tube. The aqueous phase was collected into new, clean, pre chilled tubes, where 400 μL of ice-cold isopropanol was added and samples were incubated at -20 °C overnight. The samples were centrifuged at 13.000 rpm for 15 min at 4 °C, the supernatant discarded and the pellet washed with 70 % ethanol. After a final centrifugation, the samples were allowed to air dry, and suspended in ultrapure water for posterior analysis. Each pellet was dissolved in 15 μL of RNase-free water and kept at -80 °C until use. The amount of isolated RNA and $A_{260/280}$ nm ratio was quantified using Nanodrop ND-1000 Spectrophotometer (Bonsai 06/2008 NanoDrop Technologies, Wilmington). First-strand complementary DNA (cDNA) was synthesized from 2 μg of RNA of each sample reverse transcribed (qScript™ cDNA Synthesis Kit, Quanta Biosciences) in a 40 μL reaction using thermal cycler machine.

Table II- 3. Primers used for RT-PCR analysis and expected size of the PCR products.

Target gene	Ascension No.	Primer Forward	Primer Reverse	Amplicons	Tm [°C]
Aggrecan	NM_001135	5′tga gtc ctc aag cct cct gt	5′cag tgg ccc tgg tac ttg tt	171 bp	60.4
Collagen II	NM_033150	5′ggg agt aat gca agg acc aa	5′atc atc acc agg ctt tcc ag	175 bp	57.4
Sox9	NM_000346	5′tac gac tac acc gac cac ca	5′ctc ctc aag gtc gag tga gc	217 bp	56.2
Collagen X	NM_000493	5′cag gca taa aag gcc cac ta	5′agg act tcc gta gcc tgg tt	179 bp	57.4
Collagen I	NM_000089	5′agc cag cag atc gag aac at	5′aca cag gtc tca ccg gtt tc	<200bp	51.5

GAPDH	NM_002046	5′tgc acc acc aac tgc tta gc	5′ggc atg gac tgt ggt cat gag	87 bp	60.6

bp - base pairs; *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase.

II.5.10. PCR analysis of chondrogenic markers

In chapters IV and VI real-time qRT-PCR was performed to detect amplification variations using PerfeCTa® SYBR® Green FastMix®, (Quanta Biosciences, USA) on Eppendorf Mastercycler® ep realplex gradient S machine. The relative quantification of the gene expression was calculated using the $2^{\Delta_{Ct}}$ and $2^{\Delta\Delta_{Ct}}$ method [42]. The mRNA expression levels of target genes were normalized to the average expression of endogenous housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) value. The number of cycles and annealing temperature were selected according to the manufacturer's instructions. All the primer sequences were generated using Primer3 software and acquired from MWG Biotech. More details can be found in Table 3.

In chapter VI conventional PCR was performed using a High- Fidelity DNA Polymerase (Finnzymes) and dNTP Mix (Finnzymes) as well as a primer concentration of 0.2 mM in a 35 cycle 3-step reaction. The PCR products were detected in a 1.3 % agarose gel (SeaKem®LE agarose, Lonza) using a 100 bp DNA ladder (GeneRuler™ SM024, Fermentas). Images from the gels were obtained using a UV Transiluminator (BioSpectrum AC Chemi HR 410, UVP).

II.6. IN VIVO IMPLANTATION

II.6.1. Preparation of κ-carrageenan hydrogel discs

For the *in vivo* study, described in chapter VII, the κ-carrageenan hydrogels samples were prepared as follows: the polymeric powder was mixed with distilled water under constant stirring at 60 °C to obtain a final concentration of 2 % (w/v). The polymer solution was then casted into a mould (plastic Petri dishe of 55 mm ∅) and kept at room temperature (RT) for 2-5 minutes, forming a solid gel and further stabilized by cross linking with KCl for 15 minutes. Finally the gels were washed with PBS to remove the excess of KCl present on the surface. Discs of Ø 8 ±0.01 mm x 2.5 ±0.46 mm height were then cut-off using a sterile punching device. Agarose hydrogels were used as a control material, were produced using a sterile agarose low gel temperature (800257, MP Biomedicals) 2 % (w/v) solution, heated up to 70 °C for 30 seconds, until complete dissolution. The solution was cooled at RT, decreasing the temperature and promoting gelation.

II.6.2. Preparation of cell laden κ-carrageenan hydrogel discs

In chapter VIII, κ-carrageenan hydrogels discs for subcutaneous implantation were prepared as follows: the polymeric powder was mixed with distilled water under constant stirring at 60 °C to obtain a final concentration of 1.5 % (w/v). The polymer solution (100 μl) was poured into 48 well plates and kept at room temperature (RT) for 2-5 minutes, to form gel and further stabilized with KCl for 10 minutes. Afterwards, discs of 11.5 ±0.01 mm x 1.8 ±0.24 mm height were washed with PBS to remove the excess of KCl.

The human adipose derived stem cells (hASCs) used in this assay, were enzymatically isolated 0.2 % collagenase type II (C6885, Sigma) in PBS for 45 min at 37 °C under gentle stirring as previously described in section 4.2.3. For the cell encapsulation experiments, κ-carrageenan 1.5 % (w/v) solution was added to the cells to obtain a final concentration of $5x10^6$ cells×mL^{-1}. The discs with encapsulated cells were cultured in chondrogenic differentiation medium for 7 days or freshly loaded in κ-carrageenan, just before they were subcutaneously implanted. Additional controls consisted of κ-carrageenan hydrogel samples without cells and empty defects were created. The chondrogenic differentiation medium was composed of Dulbecco's Modified Eagle's Medium- low glucose (DMEM, D5523, Sigma), supplemented with 10 % FBS (10270-106 Gibco, Invitrogen), 1% Antibiotic-Antimycotic (15240-062, Gibco, Invitrogen), ITS+1 Liquid Media Supplement (I2521- insulin-transferrin-selenium - liquid media supplement, Sigma), 17 mM L-ascorbic acid (A4544, Sigma), 0.1 M sodium pyruvate (P4562, Sigma), 35 mM L-proline (P5607, Sigma), 1 mM dexamethasone (D4902, Sigma) and 10 ng/ml of human TGF-β1 (Transforming Growth Factor-β1, 14-8348, eBioscience).

II.6.3. Subcutaneous implantation of κ-carrageenan hydrogel discs

The *in vivo* biocompatibility was evaluated by subcutaneous implantation of the hydrogels, using a total of 20 rats (12-weeks-old Wistar male rat with an average weight between 260 and 300 g), corresponding to 10 rats / time point / condition. All animal studies were performed accordingly to the national guidelines and conducted in accordance with Portuguese legislation (Portaria n°1005/92) and international standards on animal welfare as defined by the European Communities Council Directive (86/609/EEC). Surgeries were performed under general anesthesia, intraperitoneal (IP) administration route using a combination of medetomidine (Dormitor® - 0.5mg/Kg) with ketamine (Imalgene® - 75mg/Kg) and post-surgical pain control subcutaneous administration route with carprofen (Rimadyl®, 2.5-5mg/Kg) was performed for each animal. κ-Carrageenan and agarose hydrogels discs (Ø 8±0.01 mm x 2.5±0.46 mm height) were prepared as described in previous section. Under surgical sterile

conditions, two full thickness skin longitudinal incisions (about 1 cm) containing the subcutis and the panniculus carnosus (skin and smooth muscle) were performed in the dorsum of each animal (anterior and posterior incisions). Cranial and lateral oriented subcutaneous pockets were created by blunt dissection, one on each side of the incision. The hydrogel discs were inserted into these pockets (four discs of the same materials per animal), and the panniculus carnosus and the skin were carefully sutured (Figure 10). A negative control group was set for the time periods of implantation with empty pockets. As positive control group, rats were injected with LPS (1μg/g rat body weight, L4641, Sigma) 24h before the collection of exudates. After each predetermined implantation time period (7 and 15 days), each animal was induced with isofluorane and injected (IP) with an overdose of pentobarbital sodium (Eutasil® from Ceva Saúde Animal).

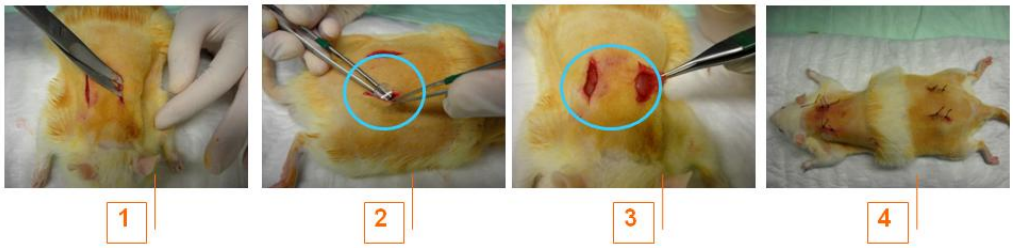


Figure II- 10. Four individual skin longitudinal incisions (1 cm) with cranial oriented subcutaneous pockets were created by blunt dissection.

II.6.4. Subcutaneous implantation of cell laden κ-carrageenan hydrogel discs

The study described in chapter VIII was designed as a pilot/preliminary study to assess the stability of the κ-carrageenan hydrogels laden with either fresh or pre-cultured hASCs, as compared to the hydrogels alone, since in the previous in vivo study it was found that the hydrogels were "dissolved" shortly after implantation. Surgeries were made on 9-week-old male Wistar rats under standard sterile conditions. Briefly, the rats were anesthetized with an intraperitoneal injection of a combination of medethomidine (30-100 μg/kg) plus ketamine (50-100mg/kg). After shaving the hair and disinfection with povidone-iodine (Betadine), a longitudinal incision was made on four distinct regions of the back of the rats. Ionic κ-carrageenan hydrogels with and without cells were implanted subcutaneously. Empty defects were used as controls. Then, the wounds were closed with a nonabsorbable polypropylene suture 4-0. All the animal experiments were performed accordingly with the National Ethical Committee for Laboratory Animals and conducted in accordance with Portuguese legislation (Portaria n°1005/92) and international standards on animal welfare as defined by the European Communities Council Directive (86/609/EEC). Each rat received the implantation of 4 constructs randomly placed on their

back. Animals were placed on a heating pad under a warming light and observed until they recover consciousness. Animals were assessed for 30 minutes following their return to consciousness to assess evidence of distress and placed in cages (2/3 per cage). Animals received analgesia preoperatively (lidocaine) and during the post-operative period (carpofen) as needed based on evidence of discomfort. This was evaluated by animal behavior, feeding, and vocalization. Sutures were removed after 7 days postoperatively and animals were euthanized by asphyxiation with CO2. The explanted tissues were fixed in 4% formalin, embedded in paraffin, sectioned and processed for histological analysis.

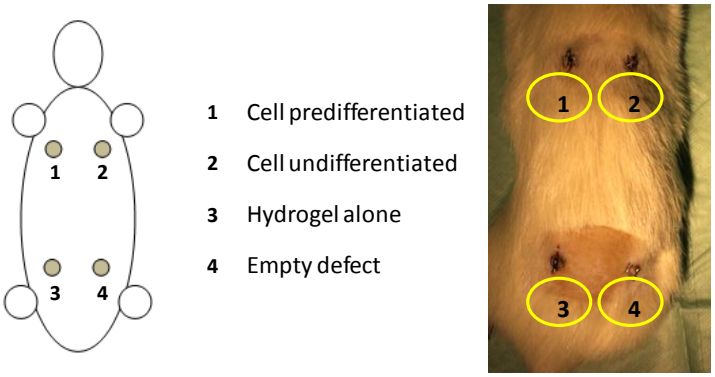


Figure II- 11. Four individual subcutaneous pockets, created by blunt dissection, were filled with different conditions.

II.6.5. Collection of explants

In chapter VII, at each time point, explants were retrieved from the four implantation sites of each animal together with the respective axillary and inguinal lymph nodes. Explants were either fixed in 3.7 % formalin for histological evaluation, or frozen for posterior PCR analysis.

In chapter VIII, the explants were retrieved from the implantation sites of each animal and fixed in 3.7 % formalin and processed under standard histological tissue processing techniques for paraffin embedding.

II.6.6. Characterization of explants

II.6.6.1. *Histological characterization*

For histological analysis, explants were subjected to standard histological tissue processing for paraffin embedding. Sections with 4 µm thickness were obtained using a microtome (HM355S, Microm, Thermo scientific) and stained with haematoxylin and eosin (05-12011/L, 05-M10003, Bio-optica) in

an automatic stainer (HMS740, Microm, Thermo scientific). H&E staining was performed based on the protocol already detailed in the section with the procedures for histological characterization (5.6.1).

In chapter VIII, sections with 4 μm thickness were obtained using a microtome and stained with haematoxylin and eosin and Masson Goldner Trichrome (MGT) staining. The last staining is recommended for connective tissue, nuclei or collagen and tree different stains were used: Weigert's iron hematoxylin for nuclei, picric acid for erythrocytes and light green for collagen. After typical hydration steps, Weigert's iron hematoxylin reagent was added to the sections and left to act for 15 minutes. Without washing, the slides were drain and picric acid alcoholic solution was added to the section for 4 minutes. After quick wash in distilled water the other reagents were put on the section to act for a total of 15 minutes. Washing in distilled water and rapid dehydration through ascending alcohols, clearing in xylene and mounting was performed as last step.

II.6.6.2. *Immunohistochemistry for CD163 and CD25 antibody*

Immunohistochemistry was performed on histological sections to assess presence/localization of different cell populations and thus tissue response to the implants, namely inflammatory cells infiltration (chapter VII). Detection of recruited macrophages and activated lymphocytes was completed using an avidin-biotin alkaline phosphatase technique. The tissue sections were dewaxed, rehydrated and the antigen retrieval was achieved by heating at 96 °C with 10 mM citrate buffer, pH=6, for 40 minutes. Sections were washed in PBS and endogenous peroxidase activity was blocked with 0.6 % hydrogen peroxide - methanol for 10 min at 22 °C. Afterwards, the permeabilization was done with Triton™ X-100 (X-100, Sigma) followed by incubation with protein block buffer for 20 min at 22 °C. Excess serum was removed by blotting and the sections were incubated overnight at 4 °C with either CD163 antibody (ED2, MCA342GA, AbD Serotec) to detect recruited macrophages or CD25 antibody (MCA494GA, AbD Serotec) to detect activated lymphocytes. After incubation, sections were rinsed with PBS for 5 min and incubated with polyclonal swine anti-mouse, goat, rabbit IgG antibody (Dako) for 1 h at room temperature. Further washes in PBS were performed prior to exposure of the sections to Vectastain elite ABC reagent (PK-6200, Vector Laboratories Ltd.) for 30 minutes at 22 °C and the substrate reaction was developed using the the 3,3′-diaminobenzidine (DAB) peroxidase substrate solution (SK-4100, Vector Laboratories Ltd.). Finally sections were counterstained with haematoxylin for 1 minute and mounted with Entellan® (4111, Inopat). Stained sections were observed under a light microscope (Reflected/Transmitted light Microscope, Zeiss Germany) and images obtained with Axion MRc5; Zeiss, camera.

II.6.6.3. *PCR ANALYSIS OF INFLAMMATORY MARKERS*

RT-PCR analysis was used to detect at the molecular level, the expression of inflammatory markers, namely *IL-1 alpha*, *IL-4*, and *IFN- gamma*. The housekeeping gene was glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) chosen as an endogenous control for the experiments. The detailed description of the sequence of the primers used is presented in Table II.4. The explanted tissue was transferred to eppendorfs tubes with 1 mL TRI Reagent® RNA Isolation Reagent (T9424, Sigma) and stored at -80 °C until processing. The homogenization of the biologic tissue was achieved using a mortar and pestle. Total RNA was extracted from samples following the manufacturer's guidelines (TRI Reagent®). The amounts of isolated RNA from samples with $A_{260/280}$ ratio between 1.6 and 2.0 were determined using Nanodrop ND-1000 Spectrophotometer (Bonsai 06/2008 NanoDrop Technologies). A predetermined amount of RNA from each sample was reverse transcribed into cDNA using qScript™ cDNA Synthesis Kit (Quanta Biosciences) using a MJ Mini™ Personal Thermal Cycler (Bio-Rad Laboratories) machine. Amplification of the sequences of interest was performed using a GoTaq® Green Master Mix (M7112, Promega) and the primers were used at a concentration of 10 μM in a 30 cycle 3-step reaction. The PCR products were visualized in a 1.7 % agarose gel (SeaKem ®LE agarose, Lonza) with a DNA marker pBR322 - Hae III (A5229, AppliChem). Images from the gels were taken using a UV Transilluminator (BioSpectrum AC Chemi HR 410, UVP).

Table II- 4. Primer pair sequences and annealing temperatures for the genes analyzed by RT-PCR on samples retrieved from *in vivo* experiments performed in a rat model.

Target gene	*Sequences*		T_m *[°C]*	*Bp*
	Sense	*Antisense*		
IL-1α	GCAAAGCCTAGTGGAACCAG	GCAGAAGGTGCACAGTGAGA	59.4	244
IL-4	TTTTGAACCAGGTCAACACCA	GTGAGTTCAGACCGCTGACA	57.4	-
IFN-γ	GCCCTCTCTGGCTGTTACTG	CTGATGGCCTGGTTGTCTTT	59.4	221
GAPDH	GGTGATGCTGGTGCTGAGTA	GGATGCAGGGATGATGTTCT	58.4	81

II.7. STATISTICAL ANALYSIS

Statistical analysis was carried out by mean ± standard error of mean. Several statistical methods were used in this thesis, accordingly to the experimental assay requirements. First, an F-test was used to ascertain about the data normality to determine equality of variance, or the data was inspected with Shapiro-Wilk test. The null hypothesis, that the means of each set were equal, was evaluated with a 95% confidence level (α=0.05). If the null hypothesis was found to be false, indicating that the means

of the different experimental treatment sets were not equal, then multiple comparison tests was performed [43].

The results indicated that at the 0.05 level, the data was significantly drawn from a normally distributed population and analysis of variance (ANOVA one way) followed by Tukey test was used to determine significant differences between groups and conditions using with OriginPro 8 program or GraphPad Prism statistic software. The difference was considered significant when $p < 0.05$ [44].

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SECTION 3. ENGINEERING ARTICULAR CARTILAGE USING κ-CARRAGEENAN HYDROGELS

Chapter III. CELL DELIVERY SYSTEMS USING ALGINATE-CARRAGEENAN HYDROGEL BEADS AND FIBERS FOR REGENERATIVE MEDICINE APPLICATIONS

CHAPTER III*

CELL DELIVERY SYSTEMS USING ALGINATE-CARRAGEENAN HYDROGEL BEADS AND FIBERS FOR REGENERATIVE MEDICINE APPLICATIONS

ABSTRACT

The present work was focused on the development and characterization of new hydrogel systems based on natural origin polymers, namely alginate and carrageenan, into different formats and with adequate properties to sustain the viability of encapsulated cells, envisioning their application as cell delivery vehicles for tissues regeneration. Different formulations of alginate and carrageenan hydrogels and different processing parameters were considered in order to determine the best conditions required to achieve the most adequate response in terms of the mechanical stability, cell viability and functionality of the developed systems. The morphology, size and structure of the hydrogels and their degradation behavior and mechanical properties were evaluated during this study. In addition to cytotoxicity studies, preliminary experiments were carried out to investigate the ability of alginate-carrageenan beads/fibers to encapsulate chondrocytes. The results obtained indicated that the different formulations, both in the form of beads and fibers have considerable potential as cell-carrier materials for cell delivery in tissue engineering/regenerative medicine applications.

Keywords: Natural hydrogels, Encapsulation, Carrageenan, Cell delivery, Chondrocytes, Cell viability.

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III.1. Introduction

Hydrogels are the basis for cell encapsulation/delivery systems, one of the most promising approaches for the delivery of cells and therapeutic agents to the site of interest, allowing a wide range of application in the regenerative medicine field [1]. During the last decade, several different hydrogels, particularly algal polysaccharides, have been used as cell-delivery matrices [2, 3]. Naturally derived hydrogels have frequently been employed because they are either composed of components or have macromolecular properties similar to the natural extracellular matrix of human tissues [4, 5]. With today's interest in novel renewable polymers, the underexploited marine red algae family belonging to carrageenan seaweeds stimulated our interest as a source of polysaccharides with innovative structure and functional properties. Many different hydrogels have been previously studied for biomedical applications, but carrageenan displays a unique potential in this field and has been very little explored until the present time. Carrageenan is a naturally occurring polysaccharide used far more widely than agar as emulsifier, gelling, thickening and stabilizing agent in pharmaceutical [6] and industrial formulations. Their relatively low cost promoted their use in environmental and commercial applications as well [7]. This hydrophilic polysaccharides resemble to some extent to the naturally occurring glycosaminoglycans (GAG's) owing to their backbone composition of sulphated disaccharides [8]. There are three main commercial classes of carrageenan namely: κappa, that has only one negative charge per disaccharide and produces strong, rigid gels (extracted from Kappaphycus cottonii), the ιota type which has intermediate sulphate content and produces soft gels that provide excellent freeze/thaw stability (extracted from Eucheuma spinosum) and lambda, which is highly sulphated, less likely to form a gel structure, but forming gels when mixed with proteins rather than water [9]. Potassium salts are essential for κ-carrageenan hydrogel in order to form this firm gel structure. As the level of potassium is increased, the resulting gel structure becomes tightly aggregated and may cause syneresis (moisture on the gel surface - the separation of liquid from a gel) [10]. Iota-carrageenan forms elastic, dry gels especially in the presence of calcium salts. The 2-sulfate groups on the outside of the ιota carrageenan molecule do not allow the helices to aggregate to the same extent as κappa carrageenan, but form additional bonds through calcium interactions. Because of the ionic nature of carrageenan, the gelling and melting temperatures are dependent almost solely on the concentration of the ions and gelation is strongly influenced by the presence of electrolytes [11]. This hydrophilic polysaccharide forms a gel with potassium and calcium ions, but also shows gelation under salt-free conditions helped by physical bonds being a thermosensitive hydrogel [12]. Thermoreversible gels, such as carrageenan, melt at elevated temperature and the gelation of the biopolymer is obtained by lowering the temperature. The temperature-induced gelation allows for an easy formation of gels

with different shapes emphasizing the versatility of the carrageenan. Contrarily to carrageenan, alginates are probably the most extensively studied and characterized hydrogels for cell encapsulation/delivery [13-15]. Besides that, alginate has a similar gelation mechanism to carrageenan and thus, in this study we have focused on the development of hydrogels composed of mixtures of alginate with different types of carrageenan. The mixture of gelled hydrocolloids, in this case alginate and carrageenan, acts synergistically as a result of the similarity in the type of the polysaccharide gelling mechanism and is expected that carrageenan favors the final properties of the mixture, providing better mechanical properties and tailored degradation profiles. The gelation reaction is produced when cations diffuse into hydrocolloid solution and specific segments of the alginate and carrageenan polymers interact with K^+ and Ca^{2+} ions [16]. In this study several methodologies were tested in order to produce a wide range of systems based on carrageenan-alginate mixtures, with different properties in terms of biological and mechanical performance. The processing methodologies used were based on ionotropic gelation and wet spinning to produce different formats like beads and polymeric fibers [17, 18]. The morphology and structure were analyzed by scanning electron microscopy (SEM), the size, shape and membrane thickness were observed by light microscopy and the mechanical properties of the obtained hydrogels were evaluated by compression tests. Furthermore, the effect of several parameters, such as pH and different ionic compositions of immersion solution, on the *in vitro* degradation of the prepared hydrogels was evaluated. The biocompatibility of the developed systems was assessed by standard cytotoxicity assays. Chondrocytes were encapsulated and cultured into the developed beads/fibres and characterized in terms of cell viability (MTS assay), cell proliferation (DNA quantification) and by histological staining. In summary, the main goal of this work consisted in the development of an efficient cell delivery system based on combining properties of two natural polymers alginate and carrageenan.

III.2. MATERIALS AND METHODS

III.2.1. Materials

Alginic acid sodium salt from brown algae (71238, Sigma, USA), κappa-carrageenan (22048, Sigma Fluka, USA), ιota-carrageenan (22045, Sigma Fluka, USA) and phosphate buffer saline (P4417-PBS Sigma, USA) were used without further purification. Calcein-AM (acetoxymethyl ester of calcein-C3099) was purchased from Invitrogen (Portugal).

III.2.1.1. *DEVELOPMENT OF ALGINATE-CARRAGEENAN BEADS*

The hydrogel beads were obtained based on a droplet technique, which consists in the extrusion of the liquid polymer from the tip of a capillary tube continuously until it achieves a critical mass and then detaches and falls into a receiving solution [19]. Alginate and carrageenan aqueous dispersions were prepared separately by dissolving each of the biopolymers in distilled water, heated up at 50 ^{0}C (for alginate) and 60 ^{0}C (for carrageenan) under constant stirring from 30 min to 2 hours until complete dissolution [20]. The mixtures/blends composed of 8.0 g alginate solution and 2.0 g carrageenan solution with concentrations varying from 2 to 3.5 % (blend of alginate with κappa carrageenan are herein designated by AK and blends of alginate with ιota carrageenan by AI), were prepared by mixing under constant stirring at 30 ^{0}C for 30 min. Previous to use, all solutions were sterilized by steam power during 30 minutes at 120 ^{0}C. The solutions were prepared just before use, in order to avoid the initial thermal degradation steps which might affect the biomaterial response. Briefly, the beads were prepared by dropping the mixture using a pump (AL-1000, Alladin Programmable Syringe Pump) from a 5-mL disposable syringe equipped with a needle of 25G inner diameter into the aqueous salt solutions stirred magnetically. The precipitating solution was composed of 100 mL of 2 % (w/v) KCl and $CaCl_2$ and the pH of the solution was previously adjusted to 7.4. After gelation at fixed conditions (30 drops/min, agitation rate 50 rpm, hardening time 30 min, 25G inner diameter of the needle) the formed beads were separated, washed and stored at 4 ^{0}C, until use. The compositions of the different formulations studied and the different parameters used for their preparation are described in Table 1.

III.2.1.2. *DEVELOPMENT OF ALGINATE-CARRAGEENAN FIBRES*

For the preparation of the polymeric fibers, it was used the same working solutions as described in the previous section for preparing the beads. The fibers were obtained by wet spinning, an easy and reproducible method, which consists in immersing the needle into the cross-linking solution. Each polysaccharide is ionically cross-linked with the corresponding cations, thus, alginate and carrageenan prefer to form a stable gel with Ca^{2+} and K^{+}, respectively. Alginate-carrageenan fibers were produced using a coagulation bath of $CaCl_2$*KCl 2%, aiming at producing 3D fiber meshes. Both κappa and ιota carrageenan can shape hydrogels with potassium and calcium salts. They have the ability to form, upon cooling and after addition of the salt, an infinite variety of gels due to development of double helices of polymeric chains into the building blocks of a three-dimensional network. The shape and stability of the hydrogels formed by extrusion techniques are determined by numerous physico-chemical factors. Experimental conditions were optimized, such as the distance between the syringe and gelation media, several inner diameters of the needle (21G, 25G and 29G); the flow rate of the

polymeric solution falling into gelation bath, the cross linker concentration and also the temperature of the system (Table 1).

Table III- 1. Conditions studied referring to the alginate- carrageenan ratio, polymeric concentration, the salts used to crosslink the gels and their concentration, different types of carrageenan (κappa or ιota), and processing parameters for obtaining the hydrogels such as hardening time, flow rate and inner diameters of the needle.

Materials	Alginate-Carrageenan ratio	Polymeric Concentration (%)	Salt type	Salt Concentration (%)	Hardening time (min)	Flow rate (drops/ min)	Inner diameters
Alginate - κappa Carrageenan	5 : 5 7 : 3 8 : 2	2 2.5 3 3.5	$CaCl_2$ *KCl	2 2.5 3 3.5	15 30 60	15 30 60	21G 25G 29G
Alginate – ιota Carrageenan	5 : 5 7 : 3 8 : 2	2 2.5 3 3.5	$CaCl_2$ *KCl	2 2.5 3 3.5			

III.2.2. Physico-chemical characterization of the developed hydrogels

III.2.2.1. *Morphological characterization*

In this work, the size, shape and surface characteristics of the developed hydrogels were examined using a stereomicroscope (Zeiss, Stemi 1000 PG-HITEC) and the images were obtained using a camera PowerShot G6, Canon. Scanning Electron Microscope (SEM - Leica Cambridge S-360, UK) was used to observe the surface and the cross section morphology of the hydrogels. For SEM analysis, samples of each condition were washed in PBS, dehydrated in a gradient series of ethanol solutions and allowed to dry completely at room temperature before sputter coating with gold (Fisons Instruments, Sputter Coater SC502, UK).

III.2.2.2. *Degradation behaviour of the prepared hydrogels*

Degradation studies were carried out by immersion of the hydrogels discs (Ø 7 ±0.01 x 10±0.02 mm height, n=3) in phosphate buffered saline (PBS, pH 7.4) and culturing medium (DMEM, pH 7.4) at 37 ^{0}C. The hydrogel were lyophilized, weighed (initial weight) before being transferred to 15 ml Falcon

tubes and soaked in 10 ml PBS or DMEM under constant agitation (60 rpm). After each selected degradation time point (final weight), namely 1, 7, 14 and 21 days, the samples were washed thoroughly with PBS to remove traces of soluble degradation products, salts, or other impurities and then dried until constant weight was achieved. The degradation solutions were changed every 7 days to restore the original level of ions activity. At the end of each degradation period, the dried samples were weighed for determination of weight loss. The extent of degradation is commonly determined by calculating the percentage of weight loss (eq 1).

$$Weight\ loss\ (\%) = \frac{Initial\ \ weight - Final\ \ weight}{Initial\ \ weight} \times 100 \qquad (1)$$

III.2.2.3. *Mechanical Testing*

Uniaxial compression tests were performed to characterize the mechanical behavior of the produced 2.5 % (w/v) alginate/κ-carrageenan hydrogels discs (Ø 15 ± 0.51 mm x 5.5 ± 0.46 mm height). The hydrogels discs of blends with ιota carrageenan type were not tested since it was nearly impossible to achieve samples with precise dimensions and thus difficult to obtain results after mechanical tests. The tests were carried out at room temperature conditions using a universal mechanical testing machine (Instron, 4505 Universal Machine). Mechanical testing was performed under compression using a crosshead speed of 5 mm/min and the results from tests conducted for at least 10 specimens. The details regarding these methods are presented elsewhere [21].

III.2.3. Biological characterization of the developed hydrogels

III.2.3.1. *Cytotoxicity evaluation*

The cytotoxicity of the constituent's released from the materials was evaluated using cell culture methods, namely MEM extraction test (72 h) in order to select the most promising and/or reject any possible systems that are cytotoxic. In all cytotoxicity tests performed, latex rubber and tissue culture plastic were used as positive and negative controls, respectively. These assays are particularly aimed at establishing the possible toxic effects of leachable released from medical polymers during extraction. The objectives of the MEM extraction test are to evaluate changes in cell morphology and growth inhibition, whereas the MTS (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium) assay determines whether cells are metabolically active [22]. The detailed method is presented elsewhere [23]. Briefly, for biocompatibility assessment of the different formulations of the developed hydrogels a mouse fibroblast-like cell line, L929 (ECACC- European Collection of Cell

Cultures, UK) was used. Cells were feed with Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS, Gibco, UK) and 1% antibiotic/antimycotic (A/B, Gibco, UK) solution. L929 cells were incubated at 37 ^{0}C in an atmosphere containing 5 % CO2 until 90 % confluence was achieved. Then, a cell suspension with a concentration of 2x10^{4} cells/ mL (4000 cells/well) was prepared and seeded onto 96-well plates. Extract of the developed materials were prepared by incubating the hydrogels with media containing serum at an extraction ratio of 3cm^{2}/ml for 24 h at 37 ^{0}C. One hundred microliter of the extracts of the hydrogel, negative control (standard culture medium), and positive control (latex rubber) were placed on 80 % confluent monolayer of cells. The absorbance of the resulting solution in each well was recorded immediately at 490 nm using an automated Multi-Mode Microplate Reader (Synergy™ HT, Bio-Tek Instruments, USA). Results were presented as OD (optical density) after extraction of the blank value (i.e; medium only) and we conducted all cytotoxicity screening tests by using six replicates. The results were expressed as percentage of cell viability calculated using the following equation:

$$Cell\ viability\ (\%) = \frac{OD\ sample}{OD\ control} \times 100 \quad (2),$$

where OD sample is the optical density obtained in the cells exposed to each extract and OD control is the OD obtained in the cells incubated with the culture medium only (negative control).

Table III- 2. Results obtained from cytotoxicity tests performed using extracts of AK and AI hydrogels with different concentrations. The results are based on the MTS test performed after 1, 3, and 7 days of culture.

Samples[a]	Day 1	Day 3	Day 7
AK2.5%	74.13 ± 0.72	40.57 ± 2.68	54.39 ± 0.78
AK3%	128.26 ± 6.89	123.00 ± 1.73	98.77 ± 4.88
AK3.5%	106.89 ± 0.62	96.23 ± 3.22	90.26 ± 5.40
AI2.5%	55.99 ± 5.39	28.59 ± 2.03	35.89 ± 0.31
AI3%	91.26 ± 5.50	86.56 ± 2.16	75.24 ± 0.89
AI3.5%	106.89 ± 0.62	96.23 ± 3.22	90.26 ± 5.40

[a]We determined the values by taking into account the fact that TCPs (negative control) corresponded to 100 %. Latex was used as a positive control of cell death and produced cell viability values that were considered to be negligible.

III.2.4. Encapsulation of ATDC5 cells into alginate/carrageenan hydrogels

Cell encapsulation experiments were performed to assess the viability of cells incorporated in the developed hydrogels and to further select/optimize the developed formulations as well as the encapsulation conditions. ATDC5 cells, a cell line established from chondrocytic cells of a mouse embryonal carcinoma (mouse 129 teratocarcinoma AT805 derived, ECACC, UK) was selected for this work, since these cells are more biologically active in a 3D environment and have been extensively studied to assess their role in producing, maintaining, and remodeling the cartilage ECM [24]. As previous experiments revealed that the shape of the hydrogel didn't affect differently the viability or proliferation of the cells herein it was decided to present results based on hydrogels with fibers shape. Prior to cell culture studies, alginate/carrageenan solutions were sterilized by heat for 30 min at 120 ^{0}C. The cells were expanded in cell culture medium (Ham's F-12 medium (DMEM-F12, Gibco, UK) supplemented with 10 % FBS (Gibco, UK), 2mM L-glutamine (Sigma), and 1% A/B antibiotic solution (Gibco, UK) at 37 ^{0}C in CO_2 incubator, until obtaining the necessary number of cells for the experiments. After trypsinization and centrifugation, the cell suspension obtained was diluted in order to encapsulate 1x10^{6} cell/ml of the polymeric solution to produce the beads/fibers as previously described. The beads/fibers with encapsulated cells were then placed into non-adherent 24 well-plates and maintained in culture for several periods of time (1, 7, 14 and 21 days) at 37 ^{0}C in a CO_2 incubator. At the end of each time of culture, the beads/fibers with the encapsulated cells were retrieved, washed with PBS solution and maintained at 4 °C in PBS for further characterization studies.

III.2.4.1. *Light optical microscopy and fluorescence staining with Calcein-AM*

ATDC5 cell morphology after encapsulation in beads and fibers was observed under inverted light microscope (Zeiss, Axiovert 40 PG-HITEC). The viability of ATDC5 cell line was investigated using Calcein AM fluorescence labeling after 21 days in culture. For this assay, the samples were washed with PBS, placed in a new well plate with 1 mL of DMEM and 2 µL of a Calcein-AM solution (at 1/1000 dilution) and incubated for 15-30 min at 37 ^{0}C in 5 % CO_2. The fluorescence staining was washed and replaced by 500 µL of formalin (10 %) for 10 min at room temperature to fix the cells. Afterwards the formalin was removed and the beads and fibers were rinsed twice in PBS and observed under a reflected/transmitted light microscope (Zeiss, Axiocam MRc5).

III.2.4.2. *Assessing the metabolic activity (MTS assay)*

The alginate-carrageenan polymers with encapsulated ATDC5 cells were cultured for 1, 7, 14 and 21 days; cell free hydrogel samples were kept in the same conditions to be used as experimental controls.

After each of the selected time points of culture, the medium was removed; samples were washed with PBS and assessed for their metabolic activity using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega). Briefly, cell–hydrogel constructs (*n=3*) were washed in PBS and placed in a mixture containing serum-free cell culture medium DMEM (without phenol red) and MTS reagent at a 5:1 ratio and incubated for 3 h at 37 ºC in a humidified atmosphere containing 5 % CO2, at the end of which 100 mL (*n*=6) was transferred to 96 well plates and the OD determined at 490 nm.

III.2.4.3. *Cell proliferation by DNA quantification*

ATDC5 cell proliferation in the hydrogels was determined using a fluorimetric double-strand DNA quantification kit (Quant-iT™ PicoGreen® dsDNA reagent, Molecular Probes, Invitrogen). For this purpose, samples that were collected at 1, 7, 14 and 21 days of culturing were transferred into 1.5 mL microtubes containing 1mL of ultrapure water. ATDC5 cell-hydrogel constructs and the hydrogel alone (without cells), used as control, were incubated for 1 h at 37 ºC in a water bath and were then stored in a -80 ºC freezer until they were tested. Prior to dsDNA quantification, constructs were thawed and sonicated for 15 min. Samples and standards (ranging from 0 to 2 µg/mL) were prepared and mixed with a PicoGreen solution in a 200:1 ratio and were placed on opaque 96-well plate. Each sample or standard was made in triplicate. The plate was incubated for 10 min in the dark, and fluorescence was measured on a microplate reader (Synergy™ HT, BioTek Instruments) with an excitation of 485/20 nm and an emission of 528/20 nm. A standard curve was created, and sample DNA values were read from the standard graph.

III.2.4.4. *Histological analysis (H&E staining)*

Hydrogels fibers samples were collected at each time point of the study and previously fixed with 10 % formalin were processed by a series of dehydration steps, embedded in paraffin and sectioned at 4 µm (HM355S Rotary Microtome, Thermo Scientific). Haematoxylin and eosin (H&E) staining was conducted in automatic staining equipment (Microm HMS 740) and then cleared in xylene (VWR) and mounted for further analysis.

III.2.5. Statistical analysis

Data of compression, degradation, MTS and DNA assay are presented as means ± standard deviations, with n=6 for each group. For statistic analysis multiple pair's comparison has been performed using One-Way ANOVA, Tukey method with a significance level of 0.05, using OriginPro 8 program. First an F-

test was used to ascertain about the data normality of the results, namely to determine the equality of variance.

III.3. RESULTS

III.3.1. Development of carrageenan-alginate hydrogel beads and fibers

This study has focused on the development of novel hydrogels based on natural-origin polymers, namely alginate and carrageenan. In order to obtain the optimum hydrogel formulation several aspects were considered, such as, the use of different types of carrageenan, ιota and κappa, different concentrations of the polymers and various concentrations of the salts were evaluated (Table 1). Parameters related to the processing method, such as flow rate and needle diameter show a direct influence in the size of the produced beads; increasing the flow rate of the polymeric solution resulted in larger bead diameter and using needles with small diameter resulted in smaller droplets. The concentration and molecular structure of carrageenan and alginate, as well as the concentrations of calcium and potassium chloride have an important effect in the gelation process. Other aspects related to the ionotropic gelation method by droplet formation, namely, droplet rates, flow rate of the viscous solution, hardening time, needle distance, needle diameter and pH of the solution were taken into account for the optimization of the gel formation. Contrary to κ-carrageenan, no spherical beads were formed with formulations based on ι-carrageenan using the selected salt solutions (Table 1). The optimum concentration of the reactants was determined using empirically classical methods. Experiments with κ-carrageenan type showed that the best bead formation were obtained by dropping 2.5 % κappa carrageenan polymer into 5 % potassium chloride solution while for ιota carrageenan type the best spheres were obtained with 3.5 % dispersion and 2 % calcium chloride. Higher concentrations of the polymer dispersion used in this study could not be dropped so easily from the needle due to the high viscosity. The same difficulty was observed with lower concentrations, which led to non-stable gels in terms of shape and structure. Alginate/κ-carrageenan hydrogels blends were formed faster than κ-carrageenan alone, due to the quick and strong reaction of alginate with Ca^{2+} ions. In addition, carrageenan basically formed softer beads with less spherical shape than the alginate-based beads. Alginate/carrageenan fibers produced by immersion of the polymeric solutions inside the chemical bath containing potassium and\or calcium ions presented the same gelling properties as in the case of beads formation.

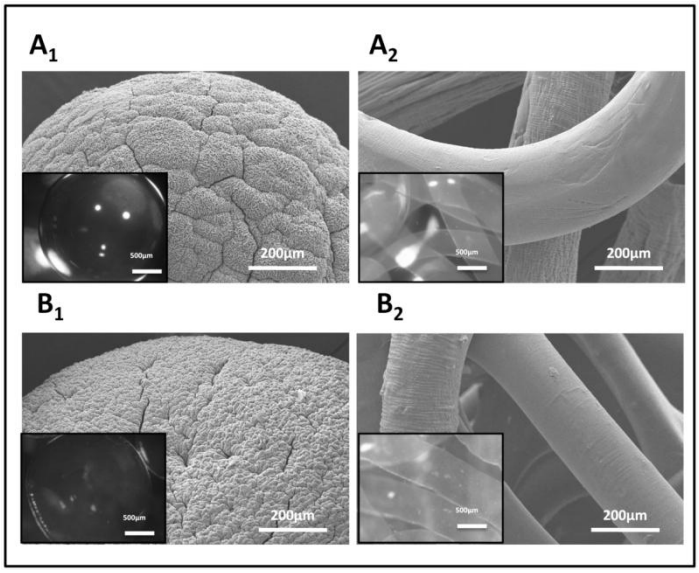

Figure III- 1. SEM micrographs of alginate-carrageenan blends surface (8:2) of beads A1 - AK 2.5 %, B1 - AI 3.5 % and fibres A2 - AK 2.5 %, B2 - AI 3.5 %; embedded images correspond to light microscopy pictures of the same samples in wet state, precipitated in 2 % (w/v) CaCl2*KCl and hardening time of 30 min.

III.3.2. Morphologic characteristics of alginate/carrageenan hydrogels

SEM micrographs of the hydrogels namely AK 2.5 % (Fig. 1 A_1 -beads and A_2 -fibers) and AI 3.5 % (Fig. 1, B_1 –beads and B_2 -fibers) showed no significant differences regarding the surface morphology. Nevertheless, blends with κappa carrageenan 2.5 % concentration and blends with 3.5 % concentration for the ιota carrageenan type led to the formation of the most stable and well defined shapes. From the light microscopy images of the beads (Fig. 1, A_1 - AK 2.5 %, B_1 - AI 3.5 %) and fibers (Fig. 1, A_2 - AK 2.5 %, B_2 - AI 3.5 %) in wet state one can observe the smooth and homogeneous surface and the well defined and stable shapes. The mean beads dimension for the AK 2.5% (8:2) formulation using 21 G needle was 2.76 ± 0.14 mm, using the 25 G diameter needle the mean dimensions was 2.16 ± 0.13 mm and when applying the needle of 29 G was 1.08 ± 0.07 mm. The mean fibres dimensions for the AK 2.5% (8:2) formulation were typically lower and ranging from 1.1 ± 0.08 mm (21 G), 0.9 ± 0.08 mm (25 G) and 0.56 ± 0.08 mm (29 G). Various complexes of hydrogels were prepared from different (5:5 and 8:2) alginate/carrageenan ratios and variations of carrageenan type namely; κappa and ιota were assessed (Fig. 2). The hydrogels were cross-linked in reaction with $CaCl_2$ and KCl and SEM (Scanning Electron Microscopy) images of cross linked and non-cross-linked hydrogels were compared. These images show that the pore size was homogenously distributed and increased with increasing carrageenan content. More than 60 % of the hydrogel volume or greater, presented porosity, indicating

that the carrageenan content is a key factor for controlling the pore size. Two different pore morphologies are obvious in the non-cross-linked, mainly due to the type of carrageenan used, κappa hydrogels exhibiting round porosity, small and circular cavities and ιota presenting longitudinal pores, large and elongated cavities. Regardless of the hydrogel composition, the pore size (ranging from 100 ~ 200 μm) of cross-linked hydrogels was smaller than the pore size of non-cross-linked ones and many pores in cross linked hydrogels were deformed by cross-linking (Fig. 2). The cross-linked hydrogel prepared from an 8:2 alginate/carrageenan mixture using κappa type, followed by reaction with $CaCl_2$*KCl, enabled the formation of an uniform hydrogel provided by a good miscibility and thus was found to be the most suitable hydrogels formulation.

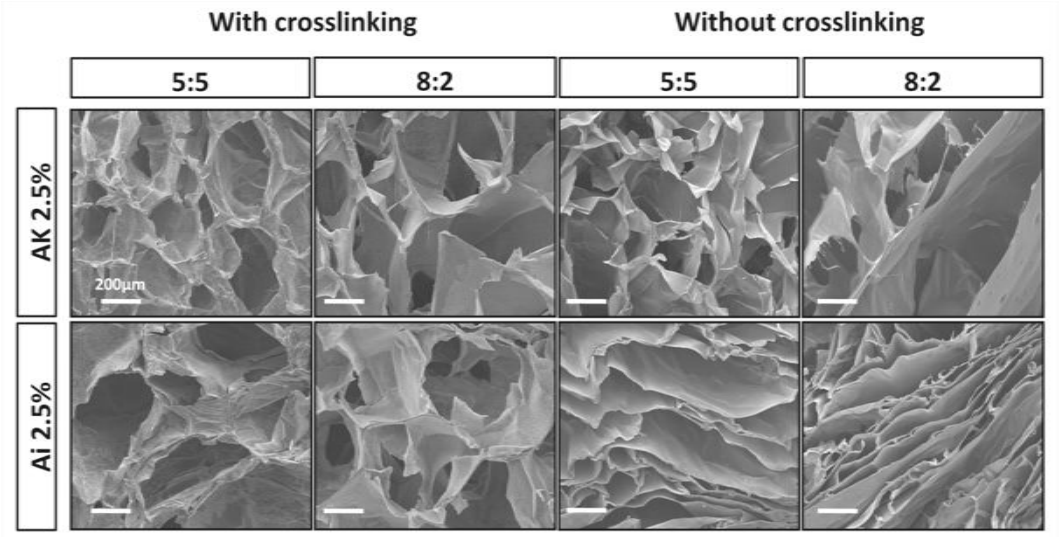

Figure III- 2. Scanning Electron Microscopy images of freeze-dried cross sections presenting the morphology and porosity of AK 2.5% and AI 2.5% hydrogel blends (5:5, 8:2 polymeric ratio, with cross-linking agent and without cross-linking, CaCl2*KCl) at 100x magnifications and 200μm.

III.3.3. In vitro degradation studies

The aim of the degradation studies was to evaluate the behavior of the developed materials in simulated physiological conditions. Therefore, the degradation of the hydrogels was monitored as a function of incubation time in PBS and DMEM at 37°C, as shown in Fig. 3. During the degradation time, most of the formulations based on ιota-carrageenan with a low concentration showed to be very unstable, disintegrating after a few days in the degradation media and therefore, only the κappa formulation was further analyzed. Moreover, it was found that the hydrogels obtained with higher polymeric concentration and a polymeric ratio of 8:2 had acceptable stability and therefore these were selected for further and detailed characterization. The polymeric concentration of the hydrogel blend

had a significant influence on the weight loss. In the first days of the experiment, the hydrogel blends with lower concentrations presented the highest weight loss rate. The hydrogels with 2 % and 2.5 % polymer concentrations lost about 35 to 45 % of their weight in the first 7 days of the experiment (Fig. 3 A). The blended hydrogels with higher concentration (3 % and 3.5 %), showed a lower weight loss rate. At day 21, the AK 2.5% formulation presents the highest weight loss. Therefore, this formulation was selected for further characterization of the degradation behavior and thus immersed in PBS and DMEM medium for up to 21 days. The results obtained from these experiments are presented in figure 3 B. Weight loss results showed fast degradation behavior in PBS solution as compared to DMEM medium.

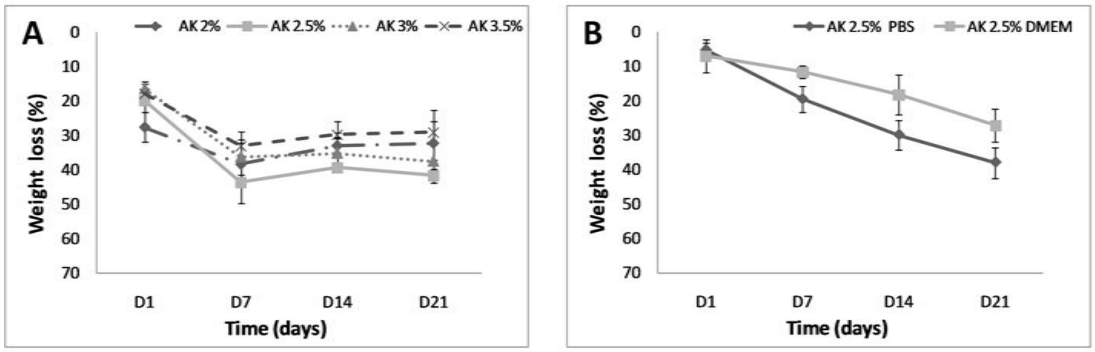

Figure III- 3. Representation of weight loss percentage vs. time of alginate/κ-carrageenan discs with different polymeric concentrations immersed in medium (A) and measurements after immersion in PBS and DMEM solutions at 37°C for AK 2.5% hydrogel blend (B). Values reported correspond to averages (n=3) ± standard deviation.

III.3.4. Mechanical characterization by compression tests

The mechanical properties of the blend consisting of alginate and κ-carrageenan 2.5 % (w/v, ratio 8:2) were examined in compression experiments. The Young's modulus for AK 2.5% (8:2) in compression was found to be 0.7 ± 0.25 MPa.

III.3.5. Biological Studies - Cytotoxicity Assays

The cytotoxicity of the components that leach out of the developed hydrogels was evaluated by a viability assay (the MTS test). Table 3 shows the results of the cellular metabolic activity obtained using extracts of the materials under study. The results obtained revealed that the extracts from the samples with a higher polymeric concentration did not affect the viability of the cell line L929. This test demonstrates the extremely low cytotoxicity levels of the alginate-carrageenan blend when using higher

concentrations of the polymers. On the other hand, the hydrogels with the lower polymeric concentrations presented a higher cytotoxicity.

III.3.6. Optical microscopy and fluorescence staining with Calcein-AM

The cells were encapsulated within the 2.5 % (w/v) alginate/κ-carrageenan hydrogel blend in beads and fibers. The light microscopic images presenting ATDC5 encapsulated in beads and fibers show a uniform cell distribution after 21 days in culture, a homogeneous cell density and smooth, well delimited shape of the hydrogels. Calcein AM fluorescence staining was conducted to assess the ATDC5 cells viability and distribution within the hydrogels. The majority of the cells were positive for calcein AM, which indicates a dominance of viable cells over a period of 21 days in culture. Three weeks post encapsulation, ATDC5 chondrocytes cells exhibited round shapes, uniform cells distribution into the hydrogel and a high cellular density (Fig. 4). These results show that both shape conditions of the hydrogels blend are not cytotoxic and that the temperature cycle used to promote the sol-gel transition does not affect cell viability.

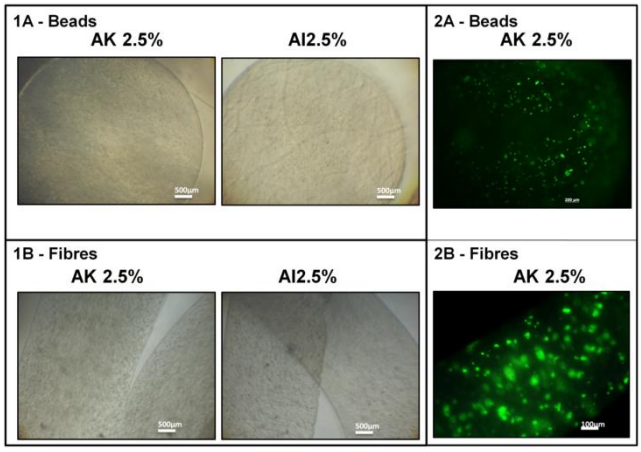

Figure III- 4. Inverted light microscopic images of ATDC5 cell encapsulated in beads and fibres (1A and 1B) and fluorescence staining, Calcein-AM (green, 2A and 2B) images depicting viable cells stained at day 21 of culture.

III.3.7. Viability and proliferation of cells encapsulated in the developed hydrogels

In this study the influence of two parameters on the viability and cell proliferation were analyzed, namely the ratio of the two types of carrageenan, κappa and ιota and also the concentration of the cross linker used for the formation of the 3D construct. Generally, from the MTS and DNA results, it was found that the ATDC5 cells remain viable and proliferate for up to 21 days of

encapsulation/culture in the 3D hydrogels developed. From the MTS assay results (Fig.5 A) we can observe that the formulations based on κappa-carrageenan enabled higher cell viability as compared to those based on ιota-carrageenan. This difference in terms of cellular response could be related to the chemical composition of ιota-carrageenan which presents more sulphated groups that κappa carrageenan, and thus the lower strength of its network [25], enables an easier leach out of cells from the tested samples. Analyzing the importance of the polymers ratio used, we can notice an increase in the metabolic activity obtained for the ATDC5 cells encapsulated in the formulation with higher alginate content, corresponding to the ratio 8:2 but not significantly different. For further biological characterization it was selected the blend with 2.5 % (w/v) alginate and κ-carrageenan with a ratio of 8:2. The influence of several concentrations of the reticulation agent ($CaCl_2$*KCl) and their biological response were investigated (Fig. 5 B and C). The results obtained from the MTS assay showed that a lower concentration of the reticulation agent has a positive effect in the cellular response especially for AK 2.5% formulation. Moreover, we can observe a higher cell proliferation with the formulation exposed to lower concentration of $CaCl_2$*KCl precipitation bath complementary to the MTS assay results (Fig. 5 C)

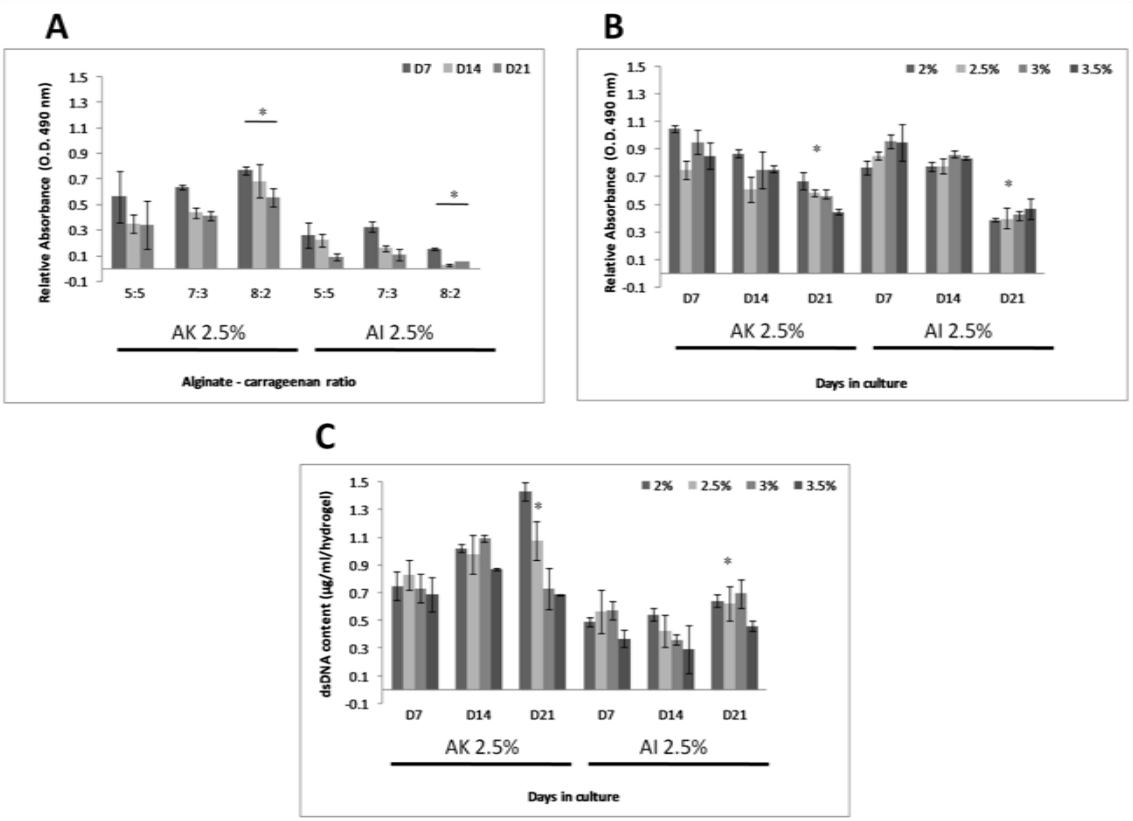

Figure III- 5. A. Metabolic activity of ATDC5 cells encapsulated in the 2.5% alginate-carrageenan with different polymeric ratio. Figure 5B present the hydrogels exposed to different concentration of the $CaCl_2$*KCl coagulation bath. The results are based on the MTS test performed after 1, 7, 14 and 21

days of culture. Figure 5C shows cell proliferation of ATDC5 cells entrapped in hydrogels blend using the two types of carrageenan with different concentration of the precipitation bath ($CaCl_2$*KCl). The results are based on the DNA quantification test performed at 7, 14 and 21 days of culture. The symbols * indicates statistical significance of the formulation AK 2.5% and AI 2.5% (ratio 8:2).

III.3.8. Histological analysis (H&E)

The morphology of the cells residing in the hydrogel blends containing one of the two types of carrageenan and with different polymeric concentration was further observed by H&E staining. Histological analysis of samples taken after 21 days of culture was performed using hematoxylin-eosin (H&E) for regular morphological cellular analysis (Fig. 6). In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. From the histological results we observed that the cells are viable, presenting homogeneous distribution and exhibit a clearly delimited nucleus over the culturing period. Encapsulated ATDC5 cells exhibit a normal morphology, some presenting small nuclei mostly in the initial culturing period, others large proliferating nuclei by 21 days of culture and in some cases, one can notice cell clustered formation in general in κappa condition. Additionally, after encapsulating ATDC5 chondrocytic cells, we observed that they were able to proliferate and maintain their typical chondrocytes morphology, namely, the lacunae aspect.

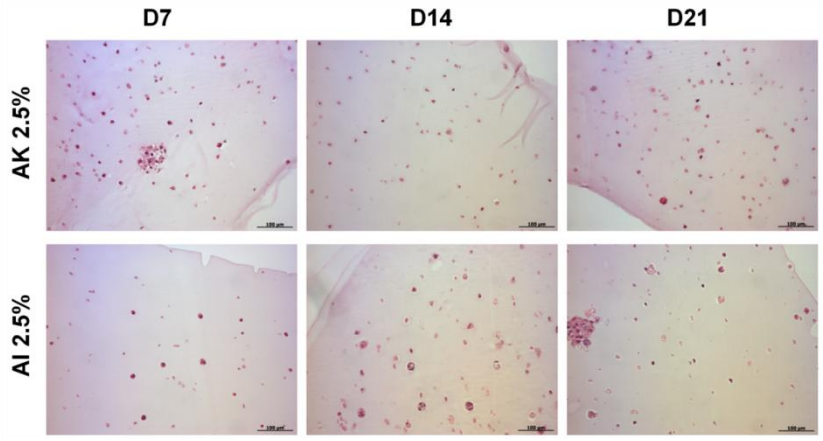

Figure III- 6. Haematoxylin Eosin staining of ATDC5 chondrocytes encapsulated in alginate/κ-carrageenan and alginate/ι-carrageenan type. The ATDC5 chondrocytes exhibited vacuoles morphology and cell clusters in κ-carrageenan formulation during the experiment, scale bars have 100 μm and images are taken at 20x magnification.

III.4. Discussion

Hydrogels can be used for cell delivery [26], as well as growth factor or drug delivery [27]. Hydrogels derived from naturally occurring polysaccharides mimic many features of extracellular matrix (ECM) and thus have the potential to direct the migration, growth and organization of encapsulated and transplanted cells during tissue regeneration [28]. In this study, a new forming biodegradable hydrogel system was developed, which may be used as a carrier for cell delivery purpose. Alginate hydrogels are fast gelation systems, hard to control, often the resulting structure is not uniform and mechanically strong and thus is difficult to achieve complex-shaped 3-D structures [29]. We expected that adding carrageenan hydrogel to the system could overcome some of these disadvantages. The hydrogels were obtained by the cross-linking of alginate and κ-carrageenan with Ca^{2+}, K^{+} ions at a stable physiological pH. Different volume ratios of the hydrogel blend were prepared for assessing the best formulation in terms of degradation profile, mechanical stability and cell behavior. It was possible to process alginate-carrageenan in different ways, enabling to obtain various structures/shapes, such as beads and fibers which can be used in different approaches/strategies for the delivery of cells. The smooth appearance of the hydrogels is clearly observed from the microscopic images of the beads and fibers in dry and wet state (Fig. 1). This demonstrates that the rigid, glassy alginate polymer can be blended with the highly flexible, amorphous κ-carrageenan polymer creating smooth morphologies and enhance the thermal stability [20, 30]. The images obtained from the cross sections of the hydrogels suggest that the morphology of the blends depends on the type of carrageenan used and this may result from the fact that κ-carrageenan gives harder gels than ι-carrageenan with $CaCl_2$ (The United States Pharmacopeia XXIII, 1995). The SEM images of the freeze-dried hydrogels also demonstrated that a higher content of *κ*-carrageenan results in the formation of smaller, more round pores and tighter network structure in the blend hydrogels (Fig. 2). A similar behavior was register in blends made with carrageenan and gelatin where the average pore size and porosity decreased with increasing κappa-carrageenan content as well, owing to the high molecular weight and viscosity of the κappa-carrageenan solution [31]. Moreover, the morphology and porosity is highly affected by the gelling agents, as compared to the degree of cross-linking with ions. However, our results showed that the type of carrageenan used in the blend composition and the alginate/carrageenan ratio are both important parameters for pore size control and shape of the hydrogel [32]. To promote the regeneration of tissues, it is important that the proposed hydrogels can delivery cells at the target site, but also maintain its structure under physiological conditions, similar to those found *in vivo,* for enough time so that they can perform a protective role for the implanted cells and thus assure their functionality [33]. In engineered hydrogel for cell encapsulation/delivery degradation can alter the diffusion of nutrients, waste and cell-material

interactions. The performed degradation studies allowed characterizing the developed hydrogels in simulated physiological conditions like PBS and DMEM immersion mediums at 37 °C by mimicking the behavior in the *in vivo* scenario. As mentioned before, it was found significant differences and systematic variation in the formation and stability of the hydrogel depending on the polysaccharide used, with κ-carrageenan being firm and rigid and ι-carrageenan being soft, elastic. Having in mind these physicochemical differences between κappa and ιota carrageenan type on the blend formation only κ-carrageenan polymer has been taken into account for degradation characterization. The hydrogel blend with low concentration revealed less stability, consequently increasing the exposure of polymer chains to water molecules, and significantly leading to water absorption enhancement and faster weight loss (Fig. 3). Also, the carrageenan component of blend would be expected to have a greater preference for water sorption than alginate because of the presence of highly polar sulfate groups in the structure [30]. Polymeric concentration influences many properties of the resulting hydrogels. In general, an increase in the polymer concentration results in a decrease in water content and mass weight loss. In other words, the hydrogels with lower concentrations present a faster degradation rate, losing almost 45 % of their weight by the end of the study. Similar degradation behavior was found for example, for hyaluronic acid hydrogel [34]. The 2.5 % alginate-κ-carrageenan hydrogels showed a slower degradation rate in DMEM medium than hydrogels exposed to PBS solution at 37 ^{0}C (Fig. 3).The divalent cations used to keep hydrogels cross-linked will be exchanged by monovalent ones, leading to the formation of less cross-linked networks, as described elsewhere [35]. This might be explained further by the higher content of ions in DMEM which interact with the hydrogels of ionic nature and also related to the possible interaction of κ-carrageenan with the proteins presented in the DMEM medium composition [36]. Concerning the mechanical properties it was found that the mechanical strength of the blends system (alginate/κ-carrageenan*)* presented values around 0.7 ± 0.25 MPa. Interestingly, the mechanical properties exhibited by the hydrogels in the wet state fall within the normal ranges of those reported for cartilage native tissue [37] and are higher than the typical values presented for hydrogels like alginate [38], chitosan [39, 40], hyaluronic acid [41]. The tensile strength of gels made from alginates reported in the literature appeared to be around 150 to 240 and 250 to 280 kPa [42]. These results clearly suggest that the compressive modulus of the developed hydrogels was improved by adding κ-carrageenan to the blends. It has been reported that the decreased sulfate content of carrageenan increases the strength of the hydrogel thus κappa carrageenan, which has less sulfated groups is expected to have better properties than ιota carrageenan [25]. Another important aspect for assessing the potential of these hydrogels for regenerative medicine applications is to evaluate their biocompatibility and their ability to sustain viability and proliferation of encapsulated cells. Since the

microstructure and high water content are very similar to that of the extracellular matrix of natural cartilage, hydrogels are expected to preserve the viability of the cells. All hydrogels produced showed a non-cytotoxic behavior. Nevertheless, lower polymeric concentrations and consequently less cross-linked networks, led to lower cell viability results, as a consequence of easier components release to the extraction medium. With increasing polymeric concentration the viscosity of the hydrogel also increases making it more stable when compared with the formulations with lower concentration, which lose their stability more easily due to the lower viscosity and cross linking. Light and live cell imaging provided a wealth of information about the biophysics and biochemistry of cells encapsulated in the hydrogels. Figure 4 presents ATDC5 cells encapsulated in beads and fibers observed under light and fluorescence microscopy. Results from samples collected after 3 weeks of culture showed the presence of viable cells in both hydrogels formats as indicated by the positive staining with Calcein AM fluorescent dye. Three formulations (5:5, 7:3 and 8:2) corresponding to the two types of carrageenan, ιota and κappa, in the blend with alginate were considered for the cell encapsulation assay. The results showed that the different ratio of alginate-carrageenan in the blend didn't affect significantly the viability of the encapsulated ATDC5 chondrocytes. From the data presented in figure 5A we can easily observe higher viability for the formulations with the κ-carrageenan type compared with the metabolic activity register for the blends made with ιota type. This cell behavior could be related to the higher sulphate composition of the ιota carrageenan type [43] . The influence of $CaCl_2$*KCl different concentration over the metabolic activity and proliferation of ATDC5 encapsulated cells showed that low concentration have higher cell viability (Fig. 5B). The DNA quantification results complemented the data obtain from the MTS test (Fig. 5C). After evaluating the influence of the polymeric concentration and the influence of the cross-linker over ATDC5 chondrocytes viability, it results clear that the first parameter is more important compared to the second. HE staining presented ATDC5 chondrocytes encapsulated within the hydrogel with typical cartilage spherical morphology (Fig. 6) predicting a potential application of the hydrogel as an injectable scaffold in cartilage tissue engineering. These results further confirmed the biocompatibility of the developed hydrogels for cell encapsulation and delivery applications. Evaluation of these properties contributed to a further understanding of the formation mechanism of hydrogels and the biological applicability of these systems. As this process of the blend hydrogel formation is simple, feasible, and usually performed under mild conditions without employing any extraneous toxic cross-linking agents, we believe that such a matrix will have potential applications as cell delivery systems, wound management, drug delivery, cartilage tissue engineering and other related biomedical fields.

III.5. Conclusions

We report herein the development of alginate-carrageenan hydrogels by a mild, friendly technique yielding on using these carriers as cell delivery systems. The morphology, degradation and mechanical behavior showed highly tunable properties, without affecting their biocompatibility. Finally the cell encapsulation assays revealed that alginate/κ-carrageenan hydrogels presented a positive cellular response, supporting the viability and proliferation during long term cell culturing. The thermo-gelling properties of such materials may be also used in new injectable systems that could be used to deliver cells, through minimally invasive procedures. In fact, all the results obtain demonstrated that alginate/κ-carrageenan can be an innovative and adequate alternative for the development of carrier systems to encapsulate cells or other bioactive agents of relevance in tissue engineering applications.

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Chapter IV. CHONDROGENIC POTENTIAL OF INJECTABLE K-CARRAGEENAN HYDROGEL WITH ENCAPSULATED ADIPOSE STEM CELLS FOR CARTILAGE TISSUE ENGINEERING APPLICATIONS

CHONDROGENIC POTENTIAL OF INJECTABLE κ-CARRAGEENAN HYDROGEL WITH ENCAPSULATED ADIPOSE STEM CELLS FOR CARTILAGE TISSUE ENGINEERING APPLICATIONS

ABSTRACT

Due to the cartilage limited self-repair capacity, regenerative medicine therapies for the treatment of cartilage defects must use a significant amount of cells, preferably applied using a hydrogel system that can promise their delivery and functionality at the specific site. This paper discusses the potential use of κ-carrageenan hydrogels for the delivery of stem cells obtained from adipose tissue in the treatment of cartilage tissue defects. The developed hydrogels were produced by an ionotropic gelation method and human adipose stem cells (hASCs) were encapsulated in 1.5 % (w/v) κ-carrageenan solution at a cell density of $5x10^6$ cells/mL^{-1}. The results from the analysis of the cell encapsulated hydrogels, cultured up to 21 days, indicated that κ-carrageenan hydrogels support the viability, proliferation and chondrogenic differentiation of hASCs. Additionally, the mechanical analysis demonstrated an increase in stiffness and in viscoelastic properties of κ-carrageenan gels with the encapsulated cells along the time in culture with chondrogenic media. These results obtained allowed to conclude that κ-carrageenan exhibit adequate properties that enable the functionality of encapsulated hASCs and thus may provide the basis for new successful approaches for the treatment of cartilage defects.

Keywords: adipose derived stem cells; cartilage; chondrogenic differentiation; hydrogels; κ-carrageenan; mechanical properties

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IV.1. INTRODUCTION

The need for tissue-engineered cartilage constructs is immense and of great clinical significance because no existing medication or surgical procedures substantially promotes the healing process of articular cartilage. Compared to other connective tissues, cartilage is avascular, aneural tissue, consisting of relatively few cells, proteoglycans and proteins and thus, when damaged due to a degenerative disease or trauma, the functional and the metabolic properties of the original hyaline tissue will hardly ever be restored [1]. Cartilage tissue engineering approaches, adopting the delivery of an appropriate type and amount of cells, with or without signaling factors, offer considerable promise as regeneration strategies [2, 3]. However, for further improvement, minimally invasive approaches and innovative cell carrier concepts should be refined, using the right biomaterials and cells sources [4]. Stem cells are the best chance for human cartilage regeneration given their ability to differentiate *in vitro* in different lineage, availability and expansion [5]. Particularly, adipose tissue has generated significant interest in cartilage tissue engineering as an abundant source of multipotent progenitor cells, which are easily acquired at high yields and can be obtained from the patient by minimally invasive procedures such as liposuction [6-8]. Hydrogels are the basis of cell encapsulation systems, one of the most promising approaches for the delivery of cells and therapeutic agents to the site of interest [9-11]. Different hydrogels have been previously studied for biomedical applications like alginate, agarose [8, 12]. In the present work we evaluated the potential use of κ-carrageenan hydrogel extracted from red algae, as a cell carrier system. κ-Carrageenan is an ionic natural polysaccharide that displays unique properties that may provide advantageous features for application in the tissue engineering field. Carrageenan is composed of large, highly flexible molecules which curl forming helical structures [13, 14], rendering them the ability to form a variety of different gels at room temperature and thus enabling a great versatility. The fully carbohydrate-based hydrogels of alternating copolymers of α-(1-3)-D-galactose and β-(1-4)-3,6-anhydro-D- or L-galactose [15] are expected to be biologically compatible, biodegradable and non-toxic. κ-Carrageenan has only one negative charge per disaccharide with a tendency to form a strong and rigid gel. Carrageenan gels in the presence of potassium ions, but has also the ability to form gels under salt-free conditions, being a thermosensitive hydrogel [16]. The mechanism of gel formation is still under discussion; nevertheless, the first step may well be double helix stabilization when K^+ is the counterion [17]. Because of the ionic nature of the polymer, the gelling and melting temperatures of κ-carrageenan are almost solely dependent on the concentration of potassium ions [18]. Thermoreversible gels, such as κ-carrageenan, melt at elevated temperature and by lowering the temperature results the gelation of the biopolymer. The temperature-induced gelation allows for the easy formation of gels in different shapes. The negative charge permits ionic interactions

with molecules like water or protein and allows a large osmotic swelling pressure. To some extent κ-carrageenan resemble to the naturally occurring glycosaminoglycans owing to their backbone composition of sulphated disaccharides [19]. At the present, there is little information about the potential use of κ-carrageenan as a new natural material in biomedical applications. Nevertheless, the results published up to date suggest that κ-carrageenan hydrogels exhibit comparable biological behavior, yet higher mechanical properties over similar hydrogels extensively used for tissue engineering purposes [20-22]. The objective of this study was to develop κ-carrageenan hydrogel aimed at application in cartilage tissue engineering. Therefore, the swelling and cytotoxicity of the hydrogels were studied, as well as their ability to sustain the proliferation and chondrogenic differentiation of hASCs Furthermore, the mechanical properties of the hydrogels with encapsulated hASCs, after different culturing time points were also evaluated. The results obtained demonstrated that κ-carrageenan hydrogels are non cytotoxic and support the proliferation and chondrogenic differentiation of encapsulated hASCs. Additionally, the mechanical properties of the cell-loaded hydrogels are maintained and even increased along culturing time, suggesting that the proposed systems provides a promising alternative to current approaches for cartilage tissue engineering.

IV.2. Materials and Methods

IV.2.1. κ-Carrageenan hydrogel preparation

An aqueous solution was prepared by dissolving the κ-carrageenan powder (22048, Sigma Fluka) in distilled water, and heating at 60 °C while stirring constantly for 2 hours until complete and homogeneous dispersion of the material was obtained. Previous to use, the κ-carrageenan solution was autoclaved during 30 minutes at 120 °C and prepared just before use. κ-Carrageenan hydrogel samples were produced using the sterilized polymeric solution with a final concentration of 1.5 % (w/v) and 5 % (w/v) potassium chloride (KCl, P5405, Sigma) as a cross linker reagent. The samples were shaped in the form of discs using cylindrical moulds and a complete gelation carried out for 2-5 minutes at room temperature to form a solid gel. After gelation, the moulds ends were cut and the cylinder hydrogel was pushed out and immersed in KCl for 15 up to 30 minutes, in order to stabilize the 3-dimensional structure. Afterwards, the gels were washed with phosphate buffered saline (PBS, D5652, Sigma) to remove the excess of KCl present in the materials. Sample discs were then sliced off from the hydrogel cylinder with a thin sterile blade into discs with dimensions of Ø 5 ± 0.01 x 2.5 ± 0.46 mm height. These samples were used for evaluating the swelling kinetics and cytotoxicity of the hydrogel.

IV.2.2. Swelling kinetics

The swelling kinetics of the developed hydrogels was studied in a PBS solution (different pH values were tested) and in culture medium (Dulbecco's Modified Eagle's medium, DMEM, supplemented with fetal bovine serum, FBS, Gibco). For this purpose, a weighed amount of each hydrogel formulation was immersed in each testing solution (PBS, DMEM = pH 7.4) and incubated at 37 °C under static conditions. The influence of the pH of the PBS solution on the water uptake of κ-carrageenan hydrogel was also analyzed. The swelling ratio was calculated using equation 1.

$$\%\ Equilibrium\ swelling\ ratio\ (\%\ ESR) = \frac{W_s - W_d}{W_d} \times 100 \quad (1)$$

W_s: weight of swollen gel after reaching equilibrium value under specified environmental conditions (PBS or DMEM); W_d: weight of the dried gel

To measure W_s, the swollen hydrogels were removed from the PBS or DMEM and immediately weighed with a microbalance after the excess of water lying on the surfaces was absorbed with a filter paper. Three samples were used for each testing condition (n=3).

IV.2.3. Cytotoxicity screening

The cytotoxicity of the hydrogels leachable was evaluated using L929 mouse fibroblast cells (ECACC-European Collection of Cell Cultures, UK) as described elsewhere [23]. Extracts of the study materials were prepared and placed in contact with a L929 cells monolayer. Briefly the extracts were obtained after 24 hours incubation of the hydrogels in complete culture medium at 37 °C and under constant agitation (60 rpm). The ratio of the hydrogel to extract fluid was equal to 3 cm^2/ml and the L929 cell seeding density was $4x10^3$ cells/well plate. In all cytotoxicity tests performed, latex rubber was used as positive control for cell death and standard tissue culture polystyrene was used as a negative control. The objective of the extraction test was to evaluate changes in cell morphology and growth inhibition, whereas the MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, G3580, Promega) test determines whether cells are metabolically active [24]. The optical density (OD) was read at 490 nm on a multi well microplate reader (Synergie Ht Izasa, Bio-Tek Instruments) and all cytotoxicity screening tests used six replicates.

IV.2.4. Human adipose derived stem cells isolation and expansion

Human liposuction aspirate samples were obtained upon patient informed consent from donors undergoing lipoaspiration procedures under a protocol established with the Department of Plastic Surgery of the Hospital da Prelada, Porto, and approved by the local Ethical Committee. All the samples were processed within 24 hours after collection. The human adipose derived stem cells (hASCs) were enzymatically isolated as previously described [25]. Briefly, the adipose tissue samples were digested with 0.2 % collagenase type II (C6885, Sigma) in PBS for 45 min at 37 °C under gentle stirring. The digested tissue was filtered with a 100 μm filter mesh (Sigma) centrifuged at 1200 rpm for 10 min at 20 °C and the cell suspension solution washed 5 min with lysis buffer to remove erytrocytes. Cells were again centrifuged; the supernatant removed and resuspended in alpha Minimum Essential Medium (α-MEM, 12000-063 Gibco, Invitrogen) with 10 % FBS (10270-106 Gibco, Invitrogen; heat inactivated), 1 % Antibiotic-Antimycotic (15240-062, Invitrogen) and sodium bicarbonate (S5761-NaHCO3, Sigma). Human ASCs were plated at a density of 3.5 x 10^3 cells/cm^2 and incubated at 37 °C in a humidified atmosphere of 5 % CO2. The unattached cells were removed after 2-3 days with repeated PBS washings. The adherent hASCs were cultured with media changes on every three days. Confluent cultures were passaged with 0.05 % trypsin (25300-062 Invitrogen). The enzymatic treatment was quenched in the presence of FBS and cell counts were done using a hemocytometer. Only passage 3 (P3) hASCs were used in the experiments.

For the cell culture experiments it was performed the isolation of adipose derived stem cells from one patient (women 31 years old) and cryopreserve them at passage 1, to avoid patient cells variability. Afterwards the cells were expanded and used until passage 3 performing three independent experiments using the same batch of cells, with the same passage. The phenotype of human adipose stem cells has been extensively investigated in previous studies using tissue sample from same anatomical site (subcutaneous), harvested with similar technique (such as lipoaspiration) and isolation following similar procedures (digestion with collagenase) by [26, 27] and also by our research group [28-30].

IV.2.5. Encapsulation of hASCs in κ-carrageenan hydrogels and in vitro cell culturing

κ-Carrageenan aqueous dispersions were prepared just before being used by dissolving the biopolymer in distilled water and then sterilized, as described in previous section. Human ASCs were detached by trypsin and centrifuged at 200 g for 7 min. Cells were resuspended in sterile PBS solution, counted using a haemocytometer and finally centrifuged. The supernatant was discarded and κ-carrageenan

1.5 % (w/v) solution was added to the cells to obtain a final concentration of $5x10^6$ cells·mL^{-1}. The mixture was resuspended for complete homogenization of the cells within the matrix. Hydrogel samples containing human ASCs were prepared using sterile cylindrical moulds and allowing to rest at room temperature for 1–2 min to form a solid gel. Discs of 5 ± 0.01 mm diameter and 2.5 ± 0.46 mm height were cut using a sterile blade. The discs with encapsulated cells were cultured either in basal or chondrogenic differentiation medium for 1, 7, 14 and 21 days, which was replaced every 3–4 days. Additional controls consisted of κ-carrageenan hydrogel samples without cells, kept in the same culturing conditions for the selected time periods. The chondrogenic differentiation medium was composed of Dulbecco's Modified Eagle's Medium- low glucose (DMEM, D5523, Sigma), supplemented with 10 % FBS (10270-106 Gibco, Invitrogen), 1% Antibiotic-Antimycotic (15240-062, Gibco, Invitrogen), ITS+1 Liquid Media Supplement (I2521- insulin-transferrin-selenium - liquid media supplement, Sigma), 17 mM L-ascorbic acid (A4544, Sigma), 0.1 M sodium pyruvate (P4562, Sigma), 35 mM L-proline (P5607, Sigma), 1 mM dexamethasone (D4902, Sigma) and 10 ng/ml of human TGF-β1 (Transforming Growth Factor-β1, 14-8348, eBioscience).

IV.2.5.1. *CELL VIABILITY AND PROLIFERATION ASSESSMENT*

At the end of each time point of the study, the κ-carrageenan samples with encapsulated hASCs were collected from the culturing plates and incubated in a Calcein AM (C3099, Invitrogen) solution of 1/1000 in culture medium for 15–30 min at 37 °C and afterward washed in sterile PBS. The stained samples were placed on a microscope slide and observed under a fluorescent microscopy (Reflected/Transmitted light Microscope, Zeiss). The proliferation of the hASCs encapsulated in the κ-carrageenan hydrogels was assessed using a fluorimetric double-strand DNA quantification kit (P7589-PicoGreen, Molecular Probes, Invitrogen). For this purpose, samples collected at 1, 7, 14 and 21 days of culturing, were washed in PBS and then transferred into 1.5 mL microtubes containing 1 mL of ultrapure water. Prior to dsDNA quantification, hASCs cell-hydrogel constructs and the sample controls (hydrogel samples without cells), were sonicated for 15 min to release all DNA from the hydrogel. Samples and standards (ranging from 0 to 2 μg· mL^{-1}) were prepared and mixed with a PicoGreen solution in a 200:1 ratio and were added to a 96-well opaque white plate. For each study material and standards, 3 samples were used for DNA assays, and triplicates of each sample were measured. The procedure followed can be found elsewhere [31] and was based on manufacture instructions. The plate was incubated for 10 min in the dark, and fluorescence was measured on a microplate ELISA

reader (Bio-Tek, Synergie HT) with an excitation of 485/20 nm and an emission of 528/20 nm. A standard curve was created, and sample DNA values were read from the standard graph.

IV.2.5.2. *Histological analysis*

Samples were collected at the end of the experiment (21 days of culture) fixed, dehydrated with the Spin Tissue Processor (Microm STP120 Inopat) and embedded in paraffin using embedding center (Microm EC350-1/EC350-2 Inopat). Sections were cut at 4 µm with the microtome (Microm HM355S Inopat) and placed on microscopy slides. Alcian blue (A3157, Sigma), Safranin O (84120, Sigma), Toluidine blue (T3260, Sigma) and Hematoxylin-eosin (H&E, MHS16, Sigma) staining were performed using the Automatic Stainer equipment (Microm HMS740 Inopat). Alcian blue, Safranin O and Toluidine blue staining were used to evaluate cartilage ECM components deposition, namely glycosaminoglycans. Alcian blue staining was performed by rinsing the sections in 3 % acetic acid (151785, Sigma) and incubating them in 1 % Alcian blue solution for 30 minutes. After that, the stain was poured off, and sections were counterstained with aqueous neutral red (861251, Sigma) for 1 min and dehydrated. The Safranin O staining consisted of staining the sections with Weigert's iron hematoxylin working solution for 7 minutes, fast green (FCF, 44715, Sigma) for 5 minutes and 0.1 % safranin O for 5 minutes. Sections were washed after each staining step, left to air dry and then rinsed in absolute alcohol. Toluidine blue staining solution was prepared by dissolving 1 % of Toluidine blue in distilled water containing 0.5 g of sodium borate (Riedel-de-Haën), followed by filtering and the sections were dipped in for 2–3 s. For H&E staining, after hydration the sample sections were colored with Papanicolaou Harris hematoxylin (05-12011/L, Bio-optica) for 3 minutes, washed in running tap water and afterwards a blue stain enhancement was performed by an immersion in 0.5 % ammonia (05002, Sigma) for 5–10 seconds. The sections were washed in running tap water and stained in Eosin-Y (05-M10003, Bio-optica) for 30 seconds. Finally all slides were dehydrated through series of alcohol immersions from 30 % until 100 % alcohol. The final step for all the staining performed was the immersion in the clearing agent Histoclear® (National Diagnostics) or xylene substitute for 1-2 minutes and mounted using Microscopy Entellan® (Merck &Co., Inc.) for later observation. Stained sections were observed under a light microscope (Reflected/Transmitted light Microscope, Zeiss).

IV.2.5.3. *Immunohistochemical analysis*

Sections of samples corresponding to all experimental conditions were obtained as described above in the histological part. Before removing the paraffin, the slides were warmed, and the antigen retrieval was performed for 20 minutes at 95 °C using 10 mM citrate buffer. Sections were washed in PBS, 10

to 15 minutes, and endogenous peroxidase activity was quenched with 3 % hydrogen peroxide (31642, Sigma) in 50 % methanol/tap water for 5 min. Afterwards, samples were washed with PBS and blocked with 3 % bovine serum albumin (A2153, Sigma) for 1 h to avoid nonspecific staining. Sections were further incubated with primary antibodies (collagen type II and collagen type I; mouse antitype II collagen - MAB1330 and mouse antitype I collagen - MAB3391, Chemicon) overnight at 4 °C, in a humidified atmosphere. Then slides were washed with PBS for 10 min each and incubated with secondary antibody from Vectastain Elite ABC Kit PK-6105 (Vector Laboratories Ltd, Peterborough) for 1 h at room temperature, again in a humidified atmosphere. The remaining protocol was performed according to that described in the in the Vector DAB Kit (Vector Laboratories Ltd). Slides were washed in water for 5 min and then counterstained with haematoxylin for nuclei visualization and finally, slides were mounted. Controls were performed using normal horse serum replacing the primary antibodies, which was also included in the kit. The samples were visualized under a light microscope and images obtained using a camera (Axion MRc5, Zeiss).

IV.2.5.4. *RNA ISOLATION AND REAL TIME QUANTITATIVE RT-PCR*

Real-time qRT-PCR analysis was used to assess the expression profile of typical markers for chondrogenic differentiation, namely *Sox9*, *Aggrecan*, *Collagen X*, *Collagen type I*, *Collagen type II* and thereby evaluate the ability of hASCs to undergo differentiation when encapsulated in κ-carrageenan and cultured with either basal or chondrogenic medium. For this purpose, total RNA was extracted from cell-hydrogel constructs using TRI Reagent® RNA Isolation Reagent (T9424, Sigma) according to the provided technical data sheet. Briefly, three samples of each condition were collected at defined time periods, washed twice with PBS, added in TRI Reagent® (800 μL) and vigorously mixed during 10 s and stored at -80 °C until the analysis was performed. At this point, 160 μL of chloroform was added, incubated on ice for 15 min and centrifuged at 13.000 rpm for 15 min at 4 °C to establish a three-phase composition in the tube. The aqueous phase was collected into new, clean, pre chilled tubes, where 400 μL of ice-cold isopropanol was added and samples were incubated at -20 °C overnight. The samples were centrifuged at 13.000 rpm for 15 min at 4 °C, the supernatant discarded and the pellet washed with 70 % ethanol. After a final centrifugation, the samples were allowed to air dry, and suspended in ultrapure water for posterior analysis. Each pellet was dissolved in 15 μL of RNase-free water and kept at -80 °C until use. The amount of isolated RNA and $A_{260/280}$ nm ratio was quantified using Nanodrop ND-1000 Spectrophotometer (Bonsai 06/2008 NanoDrop Technologies, Wilmington). After these determinations, 2 μg of RNA of each sample was reverse transcribed with qScript™ cDNA

Synthesis Kit (Quanta Biosciences) in a 40 µL reaction using a MJ Mini™ Personal Thermal Cycler (Bio-Rad Laboratories) machine. Real-Time qRT-PCR was performed to detect amplification variations using PerfeCTa® SYBR® Green FastMix®, (Quanta Biosciences) on Eppendorf Mastercycler® ep realplex gradient S machine. The analysis of the results was performed with realplex software (Eppendorf Mastercycler, Applied Biosystems). The reaction composition was the following: 10 µl of SYBR Green PCR FastMix, 2.5 µl of each forward and reverse primer asses (see Table 1) and 5 µl of diluted template with RNase-free water. The number of cycles and annealing temperature were selected according to the manufacturer's instructions. All the primer sequences were generated using Primer3 software (v 0.4.0) and acquired from Eurofins MWG Operon (Ebersberg). More details can be found in Table 1. Each gene was processed in triplicate. Human ASCs encapsulated in κ-carrageenan hydrogel cultured in basal medium were used as control cell sample. In each sample the mRNA level expression of each gene was normalized to the average expression of *GAPDH* value. The relative gene expression quantification was performed using the $2^{\Delta_{Ct}}$ and $2^{\Delta\Delta_{Ct}}$ method [32]. Three samples of each material/conditions under study were analyzed.

Table IV- 1. List of genes under evaluation, primers and annealing temperature used in the analysis of samples corresponding to encapsulated hASCs in κ-carrageenan and cultured *in vitro* for different time points.

Target gene[a]	*Forward sequence*	*Reverse sequence*	*Tm [°C]*
SOX9	5'- tacgactacaccgaccacca -3'	5'- ttaggatcatctcggccatc -3'	58.4
Aggrecan	5'- tgagtcctcaagcctcctgt -3'	5'- tggtctgcagcagttgattc -3'	58.4
Collagen X	5'- ccaggtctcgatggtcctaa -3'	5'- gtcctccaactccaggatca -3'	59.4
Collagen I	5'- catctccccttcgtttttga -3'	5'- ccaaatccgatgtttctgct -3'	55.3
Collagen II	5'- gacaatctggctcccaac -3'	5'- acagtcttgccccacttac -3'	56.4
GAPDH	5'- acagtcagccgcatcttctt -3'	5'- acgaccaaatccgttgactc -3'	57.3

[a]Real time qRT-PCR thermal cycles corresponds to the following periods: 2 minutes at 95 °C (hot start) followed by 45 cycles of 95 °C for 30 seconds, corresponding annealing temperature (noted in the table) for 30 seconds and an extension step at 68 °C for 30 seconds. A melting curve of 21 minutes and a hold step at 5 °C was performed at the end.

IV.2.5.5. *Mechanical properties of κ-carrageenan hydrogels with encapsulated hASCs*

Dynamic mechanical analysis (DMA, Tritec 2000 Triton) was conducted to characterize the mechanical properties under compression load, of κ-carrageenan hydrogel samples and hydrogels samples with encapsulated hASCs, after culturing in either chondrogenic or basal media for different time points. The samples were prepared as described above, into discs with 8 ± 0.01 mm of diameter and 2.5 ± 0.38

mm height. The study samples (n=5) were subjected to compression cycles of increasing frequencies ranging from 0.1 to 10 Hz with constant amplitude displacements of 0.1 mm using DMA equipment (Triton Technology, Nottinghamshire). Experiments were performed in simulated physiological conditions, at 37 °C in PBS medium. The frequency scans with acquisitions of 15 points per decade were performed at 37 °C with heat rate of 2 °C/min. The mechanical analysis results were presented in terms of two main parameters: storage modulus (E′- the in-phase, elastic component) and loss modulus (E″ - the out-of-phase, viscous component). The values for the compression modulus were collected at a frequency between 0.1 and 10 Hz along the 21 days of culture and damping factor is expressed for the samples cultured in chondrogenic medium.

IV.2.6. Statistical analysis

Data obtained from DNA and real time qRT-PCR analysis is presented as means ± standard deviations (n=3). Firstly, a Shapiro-Wilk test was used to ascertain about the data normality. DMA data was first inspected with an F-test for treatments, to determine equality of variance [33]. The results indicated that at the 0.05 level, the data was significantly drawn from a normally distributed population and ANOVA one way followed by Tukey test was used to determine significant differences between groups and conditions using with OriginPro 8 program.

IV.3. Results and Discussion

IV.3.1. Swelling kinetics of κ-carrageenan hydrogel

The κ-carrageenan hydrogels were obtained by a mild cross linking reaction with K^+ ions at a stable physiological pH. It was analyzed the influence of the phosphate buffer solution pH on the swelling properties of κ-carrageenan hydrogels, determining the equilibrium swelling at pH= 1, pH= 4, pH= 7.4 and pH= 11 as a function of time (Figure 1). These results showed that swelling increases with time, reaching a maximum constant value after 48 hours. In general, it was observed that the swelling ratio was increasing with increasing pH values of the PBS solution, except for the pH= 11, which registered the lowest value for the first 28 hours post immersion. The amount of absorbed water during the first 7 hours in PBS at pH= 7.4 registered the highest values, showing that the developed hydrogels exhibit the highest water uptake at physiological pH. It was also investigated the swelling kinetics of κ-carrageenan hydrogels when immersed in cell culture medium (DMEM, pH= 7.4, at 37 °C) supplemented with 10 % serum (FBS) and compared with PBS (pH= 7.4, at 37 °C), as the hydrogels

will be eventually exposed to this media during cell encapsulation and culturing (Figure 1). It can be observed that the swelling ratio in DMEM / 10 % FBS media was lower than in PBS solution.

It is known that, upon implantation of a biomaterial in the body, typically occurs a response characterized by a decrease in the local pH. Thus it was thought it would be important to know the behavior of the hydrogel at various pHs in order to predict and eventually tailor its response upon implantation, envisioning *in vivo* studies and possible future clinical applications. When increasing the pH values of the immersion solutions from 1 to values equal to the physiological pH, it was observed a significant increase of swelling ratio leading to high water absorption (Figure 1). The κ-carrageenan hydrogels soaked in medium with pH=11 presented lower water uptake revealing that the equilibrium swelling was dependent upon pH and ionic composition of the immersion mediums. As the pH of the PBS solution was above 4, the ionization of the sulphated groups of κ-carrageenan gel occurred and led to ionic repulsion. This resulted in a more hydrophilic polymer network and contributed to higher water absorption. In summary, the swelling results demonstrated that the hydrogels exhibited a pH-dependent pattern in the range of the pH that was studied. Furthermore, these results support that the amount of ions present in the medium significantly affects the hydrogels swelling properties, as confirmed by the different results obtained upon immersion in PBS and DMEM mediums [34]. It is important to note that ionically cross linked κ-carrageenan loses the stability over time *in vitro*, most probably due to an outward flux of cross linking ions into the surrounding medium [35].

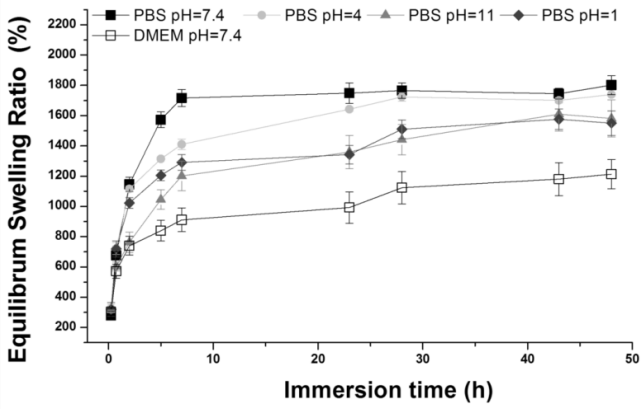

Figure IV- 1. Equilibrium swelling ratio of κ-carrageenan hydrogels as a function of time, registered after immersion in PBS solution at different pH (1, 4, 7.4, 11) and after immersion in DMEM/ 10%FBS at pH=7.4 and 37 °C. Values reported as averages (n=3) ± standard deviation.

IV.3.2. Cytotoxicity assessment

The cell behavior of hydrogel extracts was carried out as a preliminary approach to test the *in vitro* toxicity of the developed κ-carrageenan hydrogels. The cytotoxicity of κ-carrageenan hydrogels was evaluated by culturing L929 cells for 1 and 3 days with the extracts of the developed material. Figure 2 shows the results of the cell metabolic activity assessed by the MTS test performed with the hydrogel extracts. It is possible to observe that the viability levels are similar to the values obtained for the negative control (TCPs), evidencing that κ-carrageenan and possible leachable products do not exert any cytotoxicity effect on L929 cells. A significant increase in cell viability was observed from day 1 to the last time point of the assay (*$p < 0.05$). The cytotoxicity assessment of κ-carrageenan extracts was carried out as a preliminary approach to assess the potentially harmful effect of the developed hydrogels. The cytotoxicity results (Figure 2) indicate that cells were viable in the presence of hydrogel extracts for all the time points assessed. The cells in contact with the hydrogel extracts record levels of metabolic activity similar to those which were in contact with culture medium, showing extremely low cytotoxicity levels, even after long time exposure.

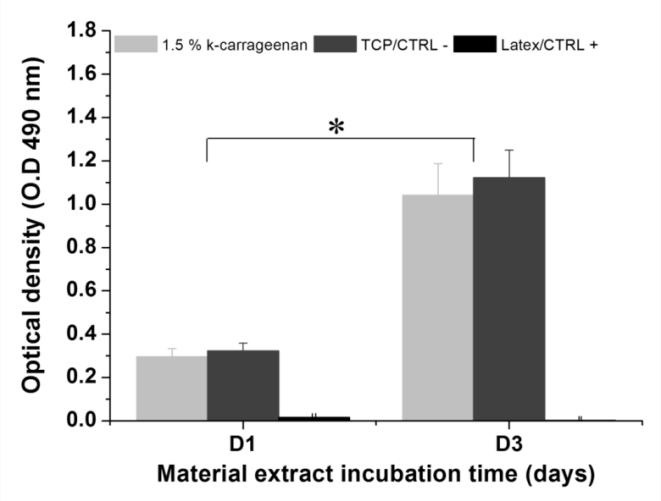

Figure IV- 2. Graphical representation of L929 cells metabolic activity after incubation with the extracts of 1.5 % (w/v) κ-carrageenan hydrogel, TCP (negative control) and latex (positive control) evaluated by MTS assay. * denotes a significantly higher metabolic activity at day 3 as compared to values obtained at day 1 of culture. Statistical analyses were conducted using one-way ANOVA for n=6 and error bars represent means ± SD.

IV.3.3. Fluorescence staining and DNA Quantification

Fluorescence staining was conducted to analyze the viability of the cells after encapsulation/culture on 1.5 % (w/v) κ-carrageenan hydrogels (Figure 3A). Calcein AM confirmed hASCs viability after *in vitro* culture, establishing that the cells were viable and homogeneously distributed inside the hydrogels. Three weeks post encapsulation, high cellular density is observed and cells are viable exhibiting round shape within the hydrogel matrix. These results confirm the temperature cycle used to promote the sol-gel transition of κ-carrageenan does not affect cell viability.

Complementary to the viability analysis along the course of the experiment, the cell content was quantified based on the dsDNA quantification (Figure 3B). Analysis of obtained DNA values sustain that from day 1 to day 7 the cell number slightly decreased for the two conditions followed by a significant increase from day 14 to day 21 (*$p < 0.05$ for basal medium and **$p < 0.05$ for chondrogenic medium). These results are also in agreement with the fluorescence viability assay (Figure 3A) showing that the κ-carrageenan hydrogel enables the viability and proliferation of encapsulated hASCs.

An important aspect for hydrogels aimed at being used as cell carriers is how the encapsulation process affects the target cells. These hydrogels have been used to encapsulate enzymes, proteins or drugs and then release them through the dissolution of the hydrogel structure [36-38] but the use of κ-carrageenan for cell delivery is still poorly exploited in the literature. It seems that a significant drop in cell proliferation from the initial time could be noticed and this behavior could be due to the fact that cells are being released from the hydrogels cell loaded systems during culturing time owing to degradation of the hydrogels. Gelation of κ-carrageenan hydrogel was induced by KCl treatment, which might initially affect the cell viability, as for fast hydrogel formation, high KCl concentration was used and the remaining KCl may influence the viability. Moreover, decreased cell viability upon cell encapsulation in hydrogels is a quite common finding, confirmed in several works reported in literature [20, 39, 40].

From the DNA assay data (Figure 3B) it was possible to sustain that until the 14 days of culture, the different culture conditions induce significant changes over hASCs proliferation. Usually stem cells proliferation decreases when the differentiation process is activated, thus the decrease in cell content could be attributed to this characteristics. The results obtained regarding the rate of proliferation of the hASCs cells, along the period that where submitted to chondrogenic differentiation medium, indicate that from day 14 to day 21 of culture, cells proliferation rate increased significantly (**$p< 0.05$). Furthermore, hydrogels loaded with hASCs cultured in basal medium presented significant lower proliferation as compared to hASCs cultured under chondrogenic differentiation medium condition. Similar studies using other hydrogels, like collagen, alginate or agarose, using either BMSCs [21] or

ASCs [20], showed similar cell behavior. All together, these results prove that κ-carrageenan enables the viability and proliferation of encapsulated human ASCs. The chemical composition of κ-carrageenan hydrogels, similar to that of the native ECM, directly affects the water content of the developed carriers which is expected to support the phenotype of chondrocytes.

In this work human adipose stem cells were encapsulated given that are of particular interest for ease way of harvest and their potential in therapies to promote cartilage repair and since there are numerous studies demonstrating that cells isolated from fat tissue can undergo chondrogenic differentiation when cultured in adequate *in vitro* conditions [41, 42]. Studies indicated that bone marrow-derived MSCs exhibit better chondrogenesis than MSCs of adipose origin, but advantages like high availability and high cell number makes adipose tissue a highly advantageous stem cell source to work with [43].

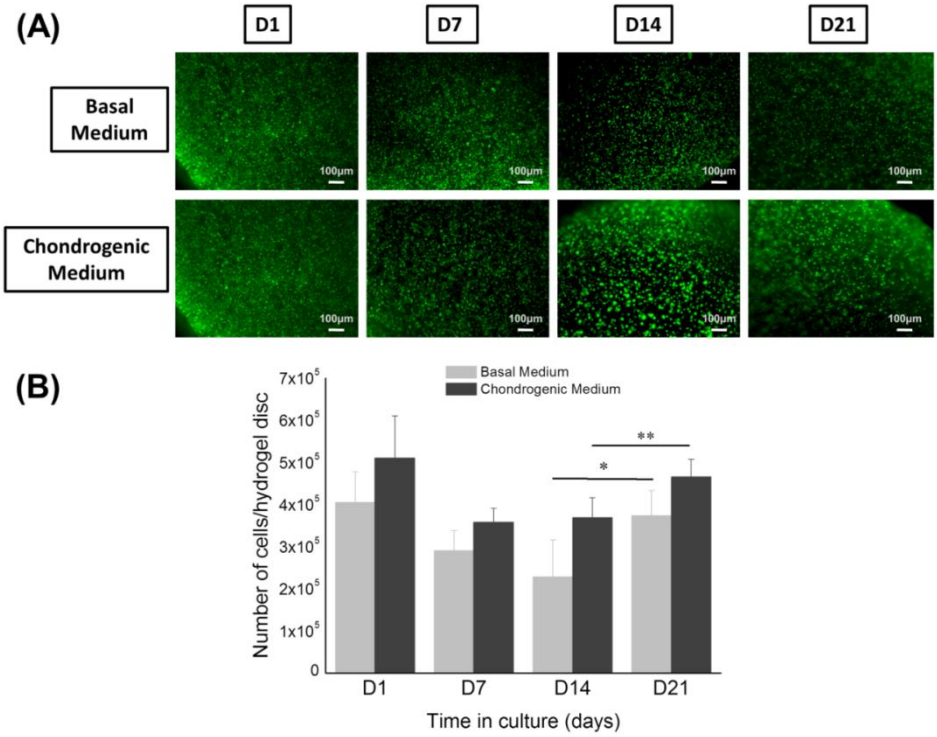

Figure IV- 3. Calcein AM staining and cell content of human adipose stem cells (hASCs) encapsulated in κ-carrageenan hydrogels and cultured for different time points in basal and chondrogenic medium. Fluorescent images showing the viability and distribution of encapsulated hASCs after 21 days of culture, magnification 5x, with 100 µm scale bar (A). Cellular content results based on the dsDNA quantification test performed after 1, 7, 14 and 21 days of culture (B). Error bars represent means ± SD. The statistics indicates that the cell number is statistically significantly higher at 21 day as compared to 14 day, for both basal and chondrogenic conditions ($p < 0.05$).

IV.3.4. Hematoxylin–eosin, Alcian blue, Safranin-O and Toluidine blue staining

Chondrogenesis occurring in the hASCs laden hydrogels can be proved by the detection of GAGs deposition and proteoglycans protein production (Figure 4) with the metachromatic staining for Alcian blue, Safranin O, and Toluidine blue. Figure 4 depicts the positive staining of hASCs encapsulated in κ-carrageenan for Alcian blue, Safranin-O, and Toluidine Blue staining, demonstrating starting deposition of proteoglycans (glycosaminoglycans, GAGs) which is commonly found in native articular cartilage ECM. The staining with Alcian Blue (Figure 4A-D) indicated concentration of acidic sulphated proteoglycans and cells appeared round with lacunae which are a distinct morphological appearance and structural characteristics specific to cartilage. For Safranin O staining we can note the similarity in the intensity of the color between nucleolus and cytoplasmic substance for the initial period of culture and the difference in color between these two for the later days in culture (Figure 4E-H). An increase in the intensity of the staining that evolved from a more orthochromatic (blue) in the initial periods of culture, to a more pronounced metachromatic (purple) staining in the later periods was noticed for Toluidine Blue staining (Figure 4I-L) similarly to mast cells found in the connective tissue. This effect was more evident in the regions where cell clusters formed in comparison to individual cells present in the hydrogels. These results show that κ-carrageenan hydrogel is not only a laden system for the cell, but is supporting cells functionality, namely the chondrogenic differentiation, mimicking ECM properties observed by the interaction and cell spreading within the hydrogel. By the end of the culturing time, setting and partial chondrogenesis is taking place and longer time in culture will promise the fully differentiation of the human derived adipose stem cells. κ-carrageenan hydrogels maintain the chondrocyte phenotype and promote cartilage-specific ECM deposition even without growth factor stimulation as subsequently it was detect chondrogenic features of the cell laden hydrogel cultured in basal medium, concluding that the chondrogenic induction was hydrogel dependent.

H&E stained sections, corresponding to Figure 4M-P, picture the typical tissue morphology, cell nuclei are stained dark blue, whereas the cytoplasm and ECM have varying degrees of pink staining. At the end of the first week of culture it is possible to observe a homogenous distribution of the cells with smaller size basophilic nucleus (blue) when in the following weeks cells assumed a more rounded morphology with cytoplasmic eosinophilic substance (pink) around the nucleus.

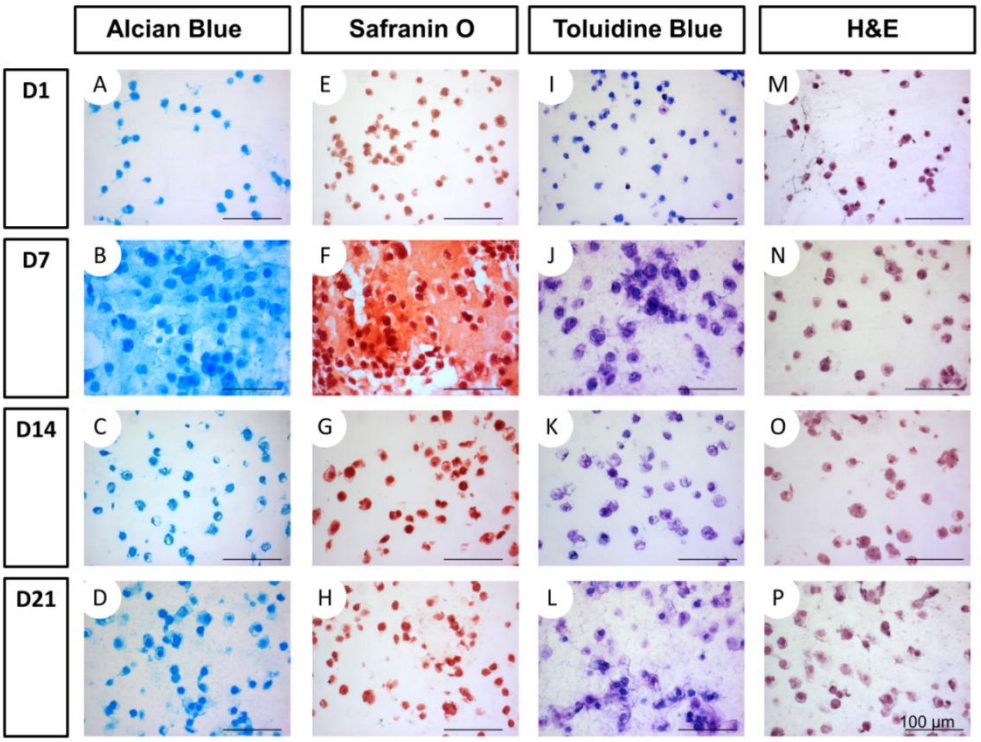

Figure IV- 4. Optical microscopy images of histological sections obtained from κ-carrageenan hydrogels with encapsulated hASCs culture in chondrogenic medium collected after several periods of culture and stained with Alcian Blue (A-D), Safranin O (E-H), Toluidine Blue (I-L), and with hematoxylin-eosin (M-P). The scale bar corresponds to 100 µm and the magnification used was 40x magnifications.

IV.3.5. Immunohistochemistry and real time qRT-PCR

To further evaluate the chondrogenic differentiation of hASCs encapsulated in 1.5 % (w/v) κ-carrageenan hydrogels, immunohistochemical analysis of specific proteins like type II collagen and type I collagen was carried out. Figure 5A-D and Figure 5E-H show that the cells were positive for collagen type II and type I during the studied culture times and show that the staining intensity increased with increasing days of culture, in particular for collagen type II. Differences were found at the histostructural level between the two proteins evaluated, with collagen type I being detected at early culture times, whereas positive staining for collagen type II was detected for the latest culture times. However, chondrogenesis is typically a progressing process, from a morphogenetic phase to a cytodifferentiation phase of development via a number of precursor stages to the mature chondrocytes [44]. Since a heterogeneous population was used it is expected to have also positive staining for collagen type I. With time, the cells enlarged and secreted a dense organization around the nucleus. On close inspection, cells from 21 day culture were spherical in shape with cytoplasmic depositions. Although most of the cells had retained the round shape typically expressed by chondrocytes that

populate the deeper layers of articular cartilage [45], cells lying closer to the surface were flattened. No positive labeling was found in the negative control sections given the absence of the key protein markers (Figure 5I-L). The presence of a metachromatic-staining matrix, the chondrocytes like appearance of the cells and the detection of type II collagen suggest that the tissue generated by these cells is similar to the native cartilage.

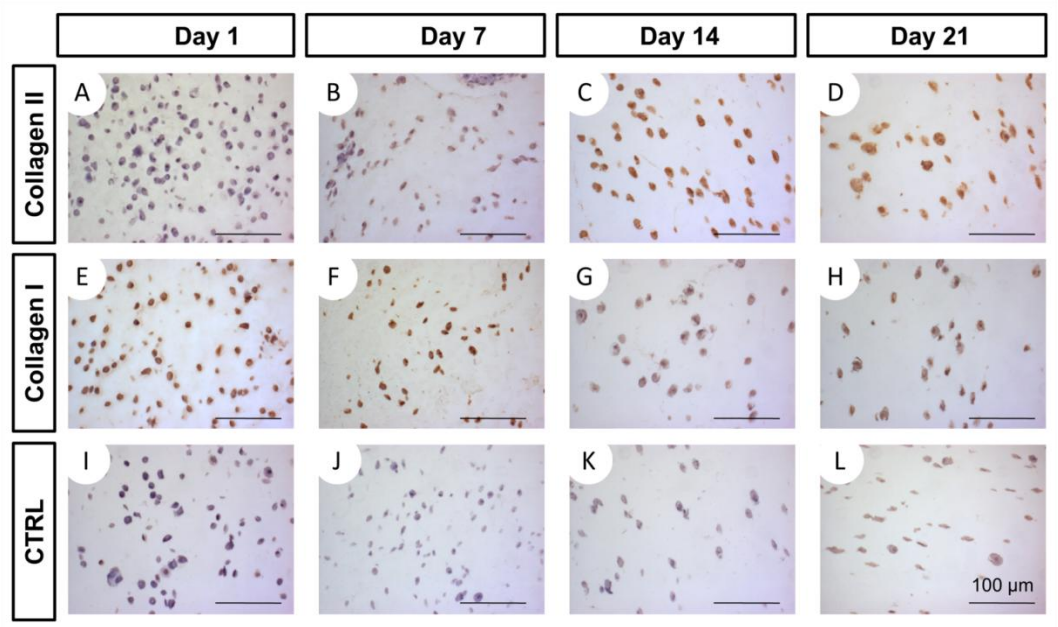

Figure IV- 5. Light microscopy images obtained from immunohistochemical staining of hASCS encapsulated in κ-carrageenan hydrogels after 21 days of culture for collagen type II (A-D) and for collagen type I (E-H) and negative controls (I-L). Scale bar corresponds to 100 µm and 40x magnification.

The chondrogenic differentiation of encapsulated hASCs was additionally evidenced by the results obtained from real time qRT-PCR analysis. The analysis allowed assessing the expression, at a molecular level, of several important typical chondrogenic markers for the hASCS encapsulated in the κ-carrageenan hydrogels (Figure 6). Sustained gene expression levels were registered for *Sox9* (*$p < 0.05$) and A*ggrecan* (#$p < 0.05$) increasing significantly with time from day 14 until the end of experiment. Actually, *Sox9* was up regulated to values close to 30 folds from 1 to 21 days. *Collagen type II* and *Aggrecan* are considered to be the two major and most important constituents of hyaline cartilage ECM since the functionality of this tissue relies mostly on the presence of these components (Figure 6). *Collagen type II* gene expression decreased from day 7 to day 14 of culture, but is significantly increased from day 14 today 21 (**$p < 0.05$). The expression of *Collagen type I*, unlike

Collagen type II, register an increase between 7 and 21 day of culture with 2 folds values (&p < 0.05). At the end of culture it was possible to notice that the mRNA expression of *Sox9* gene remains highly, as well as the gene expression of *Aggrecan* and of *Collagen type II*. It might be mentioned that *Collagen type X*, related to the hypertrophic stage of chondrogenic differentiation, register a slight increased expression over the culturing time studied. In general, from the analysis of genes expression after 3 weeks of culture, different stages in the cell life can be distinguished. Several works reported in literature [46, 47] investigating the chondrogenic differentiation of stem cells, state that the starting of chondrogenesis is around day 14, when the cells start to express chondrogenic markers as we reported in this study as well. The time-frame of the study, namely 21 days of culture was selected with basis on previous works with similar aims, using encapsulated stem cell in hydrogel for chondrogenic differentiation [21, 48, 49]. Though it will be interesting to verify the outcomes in a long time experiment, envisioning the clinical application of the proposed strategy, we are mostly concerned with the ability of the hydrogels to guarantee early pre-differentiation as a long term *in vitro* culturing before implantation is not interesting in a clinical/industrial scenario.

Sox9 gene expression was prematurely up regulated, which indicates early onset of chondrogenesis [50]. *Sox9* is an important regulator of the chondrocyte phenotype [51] and controls the expression of *collagen II*, a well known marker of the ECM of cartilage, which is usually expressed in the end stage of chondrogenic process of differentiation [52]. In this system, hASCs were likely stimulated down the chondrogenic pathway by TGF-β1 growth factor present in the chondrogenic medium. These findings indicated that hASCs can be successfully differentiated into chondrocytes when encapsulated in κ-carrageenan.

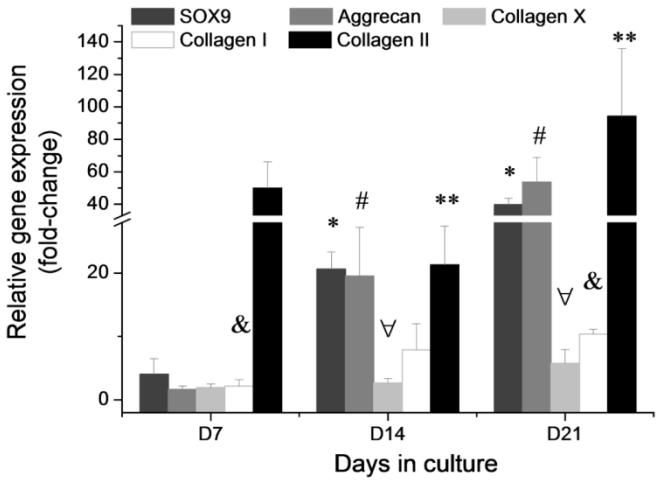

Figure IV- 6. Relative expression of chondrogenic specific genes, namely, *SOX9*, *aggrecan*, *collagen X*, *collagen type I* and *collagen type II*, based on the mRNA produced by the encapsulated human ASCs after 7, 14 and 21 days of culture. The expression of these genes was normalized against the housekeeping gene *GAPDH* and calculated by the ΔCT method. Statistical analyses were conducted using one way ANOVA for n=3; $p < 0.05$. Error bars represent means ± SD.

IV.3.6. Dynamic mechanical analysis (DMA)

Compressive modulus is a particularly important parameter to consider in cartilage tissue engineering as native cartilage is subjected to movement (i.e., walking, running), loading and unloading are transient events, occurring in <1s. Hence, the mechanical response of cartilage should be measured under dynamic cyclic conditions at a functionally relevant frequency, i.e., in the range of 0.5–3 Hz [53, 54]. The investigation of the viscoelastic properties of biodegradable hydrogels systems may be of great interest because one can not only simulate the physiological dynamical loading, but can also access relevant fundamental information at the molecular level, both from a structural and dynamic perspective [55]. Moreover, because of its complex structure and interactions of its biochemical constituents and due primarily to fluid flow through the solid matrix, cartilage behaves mechanically as a viscoelastic solid [56].

DMA allowed determining the mechanical properties of κ-carrageenan hydrogels with encapsulated hASCs after different culturing times (1, 7, 14 and 21 days) in either basal or chondrogenic medium (as compared to κ-carrageenan hydrogels without cells), while immersed in a PBS solution at 37 °C and throughout a physiological relevant range of frequencies. Storage (elastic) and loss (viscous) components of the complex modulus were determined and are presented in Figure 7. The elastic modulus (E') curve shows how the stiffness of the polymeric material changes with time in culture

(Figure 7A). At a frequency of 1 Hz, the storage modulus of the hydrogels was estimated to be 0.23 MPa for hASCs in basal medium, 0.22 MPa for cells cultured in chondrogenic medium and 0.17 MPa in κ-carrageenan gels alone by day 21, revealing the elastic nature of these gels. In this work the storage modulus (E') and phase angle or damping (tan δ) were monitored as a function of the frequency for the different hydrogels. The storage modulus curves present how the stiffness of the polymeric material changes with frequency (Figure 7B-D). Increase in E' is observed for increasing frequencies from 0.1 and 10 Hz, which suggests that the hydrogels exhibit stiffer behavior for higher frequencies. With the application of high frequencies to the hydrogel, the material becomes glassy and solid-like; at very low frequencies, the polymer exhibits a more liquid-like or rubbery response. The storage modulus of the hydrogel without cells presented variation between initial to final time points, suggesting that the hydrogels become softer, possibly due to a loss of the cross-linking ions or to a loss in gel stability due to thermal factors, as it is a thermoreversible and ionic hydrogel. The elastic modulus of κ-carrageenan hydrogels was improved with encapsulation of hASCs and along the time in culture (Figure 7A). Similar behavior was found for agarose or alginate hydrogels with the same type of cells and similar culturing conditions [20]. Nevertheless, κ-carrageenan with encapsulated hASCs exhibits a compression modulus approximately 10 x higher than that reported for agarose and alginate hydrogels in that study [20]. Hydrogels possesses also damping capability that may be useful to dissipate cyclic mechanical energy that is imposed in an implantation scenario. Damping is the term used for the general tendency of vibrating materials or structures to lose some elastic energy to internal heating or external friction, it tell us how good a material will be at absorbing energy [57]. The corresponding graphs are shown in Figure 7E. An increase in tan δ is observed from 0.1 to 10 Hz for day 1 for chondrogenic medium condition, suggesting a higher dissipation capability of κ-carrageenan hydrogels at higher frequencies (Figure 7E). At the beginning of culture we can notice an increase in tan δ values but after 21 days in culture, we can observe a decrease for increasing frequencies, which is related to higher viscous component of hydrogel biomaterial.

During the 21 days in culture, DMA analysis indicated an increase in storage modulus and in viscoelastic properties of κ-carrageenan gels with encapsulated cells suggesting an increase in the stiffness possible due to the ECM production. It is believed that the subsequent deposition of ECM components within the gel constructs was facilitated by the gradual degradation of the gels. At day 21 the statistical analysis indicated that the means difference is significant at the 0.05 level between the cell encapsulating hydrogels compared to the cell-free hydrogel control. The mechanism behind increasing the stiffness of the hydrogels laden with cells, likely involves more than the cell load as it is a result of the cell-mediated interactions and deposition of matrix with time. In addition, stem cells can

proliferate as well as differentiate but as cells differentiate their rate of replication usually decreases and by the end of 21 days there isn't any difference in terms of elastic modulus values between the cells exposed or not to the differentiation media. It has been proved that the biomechanical properties of articular cartilage greatly depends on composition, density of its ECM and interstitial fluid flow (water and solutes) [58]. So, cells encapsulation and ECM deposition may result in progressive increase of the mechanical properties of the 3D structures, as shown before for the cell-hydrogel construct [59].

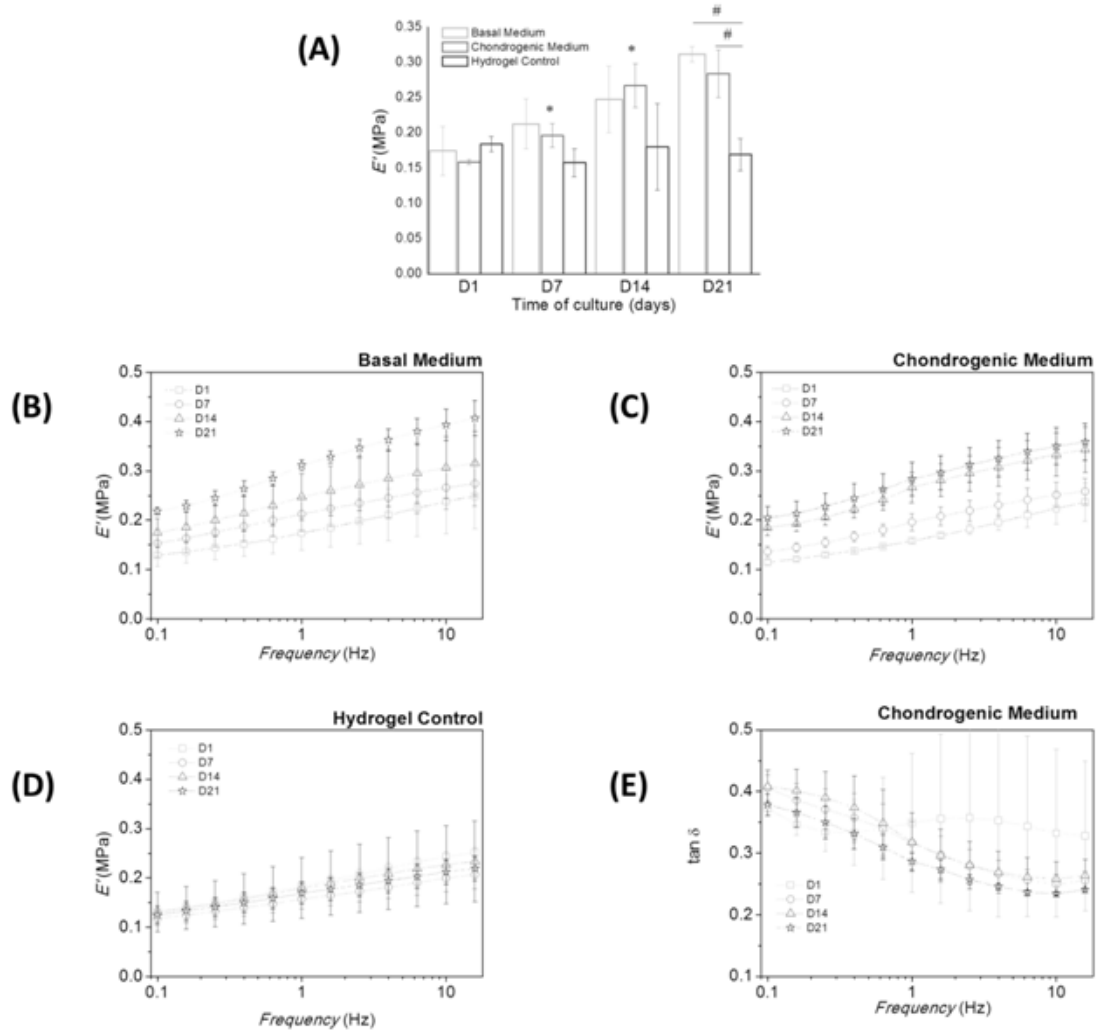

Figure IV- 7. Storage modulus (E') obtained from dynamic mechanical analysis upon compression of plain hydrogels and hydrogels with encapsulated hASCs, cultured in basal and chondrogenic medium, tested at a frequency of 1 Hz (A). The frequency sweep test presents the storage modulus in a logarithm scale at frequencies between 0.1 to 10 Hz of κ-carrageenan hydrogels with hASCs cultured in basal medium (B), hydrogels with hASCs exposed to chondrogenic medium (C) and hydrogels control (D). The damping factor (tan δ) is displayed upon compression over a period of 21 days for

chondrogenic condition (E). Values reported correspond to an average of five test samples (n=5), ± standard deviation.

The characterization at the molecular level correlates with the mechanical properties developed by these systems as at day 14 we observe a decrease in *Collagen type II* chondrogenic marker (responsible for the tensile property), at the same time point there is an upregulation of *Aggrecan* gene expression (responsible for the compressive properties of the hydrogel), as these markers are interplaying together and are being expressed differently during the chondrogenic differentiation. These values mimic the mechanical properties found in articular cartilage and they are higher or within the range of values found for other hydrogels used in similar cartilage regenerative approaches [60, 61]. Human articular cartilage possesses significant mechanical properties, with a compressive modulus of 0.79 MPa, a shear modulus of 0.69 MPa, and a tensile modulus varying between 0.3 and 10.2 MPa [62]. Evaluation of these properties contributed to a further understanding of the potential of these hydrogels for the target application.

Considering the suitable mechanical properties, together with the simplicity and reproducibility of the preparation process for the κ-carrageenan hydrogel formation, which is performed under mild conditions without employing any extraneous toxic cross linking agents, it is possible to conclude that such matrix has potential applications in cartilage tissue engineering applications. To further enhance the quality of engineered cartilage tissues formed from encapsulating hASCs within this system, one may optimize the release rates of supplemental growth factors incorporated in the hydrogels. It is also possible that host cells could be recruited to the gel, by release of the growth factors, participated in cartilage formation.

IV.4. Conclusions

This work presents κ-carrageenan as a potential hydrogel for cell delivery with application in the regeneration of cartilage. In summary, the results obtained using κ-carrageenan hydrogels as cell loading matrix, prepared via ionic cross linking reaction, demonstrate that this system could be an alternative cell delivery hydrogel systems. Human adipose stem cells encapsulated in κ-carrageenan hydrogels remain viable, proliferate and differentiate into the chondrogenic lineage. Furthermore, κ-carrageenan hydrogels with encapsulated hASCs were able to achieve mechanical properties, under compressions, in the range of those reported for native cartilage. These promising *in vitro* results

should be further analyzed in animal model experiments designed to address fundamental questions concerning the *in vivo* degradation of the hydrogel and the possible *in vivo* differentiation of encapsulated cells.

Acknowledgments

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Chapter V. CHONDROGENIC PHENOTYPE OF DIFFERENT CELLS ENCAPSULATED IN K-CARRAGEENAN HYDROGELS FOR CARTILAGE REGENERATION STRATEGIES

CHAPTER V*

CHONDROGENIC PHENOTYPE OF DIFFERENT CELLS ENCAPSULATED IN Κ-CARRAGEENAN HYDROGELS FOR CARTILAGE REGENERATION STRATEGIES

ABSTRACT

Engineering articular cartilage substitutes using hydrogels with encapsulated cells is an approach that has received increasing attention in recent years. Hydrogels based on κ-carrageenan, a thermoreversible natural origin polymer has been recently proposed as a new cell/growth factors delivery vehicle for regenerative medicine. In this work we report the potential of such hydrogels encapsulating either human adipose derived stem cells (hASCs) or human primary (nasal) chondrocytes (hNCs) or a chondrocytic cell line (ATDC5) for cartilage regeneration strategies. The *in vitro* cellular behavior of the encapsulated cells within κ-carrageenan hydrogel was analyzed after different culturing periods using biochemical assays, histological and real time RT-PCR analysis. The 3 types of cells encapsulated in κ-carrageenan hydrogels showed good cellular viability and proliferation up to 21 days of culture and the cell laden hydrogels showed to be positive for specific cartilage markers. In summary the results demonstrate that hASCs embedded in κ-carrageenan hydrogels proliferate faster and exhibit higher expression levels of typical cartilage markers as compared to hNCs or ATDC5 cells. Based on this data, it is possible to conclude that κ-carrageenan hydrogel provides a good support for culture and differentiation of encapsulated cells and that hASCs may provide an advantageous alternative to primary chondrocytes, currently used in clinical treatments of cartilage defects/diseases.

Keywords: Adipose stem cell, Cartilage, Cell encapsulation, Chondrocytes, κ-carrageenan, hydrogels

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V.1. INTRODUCTION

The limited capacity of adult articular cartilage for self-repair is well recognized and is explained by being an avascular and aneural tissue that consists of relatively few cells which cannot migrate, specially mesenchymal stem cells cannot be recruit or get easy access to damaged areas [1, 2]. Currently, clinically used therapies involve either surgical technique or autologous chondrocytes transplantation which are generally successful in terms of pain relief and improved function but do not restore completely the articular cartilage function [3]. Although the use of autologous chondrocytes have the advantages of avoiding possible immune rejection or disease transmission, their wider application presents several limitations associated with the harvesting and *in vitro* proliferation procedures. The isolation of autologous chondrocytes requires an additional surgical intervention to harvest cartilage tissue, which often results in donor site morbidity. Moreover the amount of articular cartilage tissue is limited, even if retrieved from a different site location, such as the nasal septum [4]. In addition, the *in vitro* chondrocyte proliferation must be narrowed since these cells easily dedifferentiate in monolayer cultures and because of their low capacity to produce stable cartilage upon *in vivo* implantation with a tendency to form fibrous cartilage [5]. Since adult stem cells can be isolated from various tissue sources, having the ability to proliferate and differentiate along multiple lineage pathways in a reproducible manner, they have received great attention as an alternative resource to autologous chondrocytes, overcoming the limited number of primary chondrocytes and proliferation capacity [6, 7]. Thus, many studies have been conducted to evaluate the use of adult stem cells in cartilage tissue engineering, most of them focusing on the use of bone marrow stem cells (BMSCs). Recently, adipose derived stem cells (ASCs) have been regarded as good candidates for the repair and regeneration of articular hyaline cartilage due to their availability, long term cell viability and multilineage differentiation potential, including into the chondrogenic phenotype [8]. In fact, estimated yields of stem cell are in the range of 400,000 stem cells per milliliter of adipose tissue [9], resulting in an up to a 300-fold increase in cell number from 100 grams of adipose tissue as compared to 100 mL of bone marrow aspirate [10]. Nevertheless, in approaches consisting on the direct implantation of cells into the injured cartilage area, there are some challenging issues such as the leakage of transplanted cells from the cartilage defects leading to a low cell retention, and donor site morbidity [11]. Thus, three-dimensional vehicles like hydrogels have been proposed to deliver high amounts of cells with a well-maintained phenotype, to facilitate cell retention and assist mechanical stability of the transplantation site [12]. In addition, the use of a hydrogels might provide important features for cells functionality due to their high tissue-like water content, closely mimicking the natural environment in

the body, enabling efficient transport of nutrients and waste [13] and the possibility to control the release pattern of signaling molecules by incorporating them into the hydrogel by physical and/or chemical means. Various studies have demonstrated the successful use of natural polymeric hydrogels for cell encapsulation and *in vitro* culture purposes such as hyaluronic acid [14], agarose [15], collagen, alginate [16, 17] and chitosan [18]. However, their mechanical properties are often insufficient for the stability of the tissue-like construct transplantation. Furthermore, hyaluronic acid promotes early inflammation which then is rapidly metabolized *in vivo* by free radicals and hyaluronidase [19]. Alginate has high molecular weight and therefore cannot be readily eliminated by the body [20], and chitosan induce rapid bone regeneration at initial stages [21]. κ-Carrageenan, an ionic hydrogel proposed recently for cartilage regeneration approaches, which forms a gel with potassium ions, but also shows gelation under salt-free conditions, has demonstrated advantageous properties compared to other systems currently proposed for cartilage tissue repair. In this study, we evaluated the ability of κ-carrageenan hydrogels to support cellular functionalities, comparing simultaneously the potential of using human derived adipose stem cells (hASCs) versus primary human chondrocytes, in cartilage regeneration strategies. Additionally, ATDC5, a murine well-characterized chondrogenic cell line, routinely used as a model system to study the chondrogenic process, was used as a control. The three different cell types were encapsulated in κ-carrageenan hydrogels and cultured for up to 21 days. The cells response was evaluated in terms of metabolic activity (MTS assay), proliferation (DNA), presence of glycosaminoglycans (typical histological staining, Alcian blue and Safranin O) and by the expression of specific cartilage markers (namely, Sox9, aggrecan, collagen type I, collagen type II and collagen type X) using real time qRT-PCR analysis. The results obtained suggest that, κ-carrageenan hydrogel enables beneficial cellular response supporting extracellular formation and sustaining the chondrogenic phenotype. Moreover, encapsulated hASCs exhibit the highest proliferation rates and highest levels of chondrogenic markers expression, encouraging the use of these cells and the κ-carrageenan hydrogels as cell delivery carriers for applications in the regeneration of articular cartilage defects.

V.2. Materials and Methods

V.2.1. Materials

κ-Carrageenan powder (22048), potassium chloride (KCl, P5405), phosphate buffered saline tablets (PBS, P4417), Dulbecco's Phosphate Buffered Saline (D5652), Dulbecco's Modified Eagle's Medium-low glucose (D5523), sodium bicarbonate ($NAHCO_3$, S5761), dexamethasone (D8893) were purchased

from Sigma Aldrich (Germany). Fetal Bovine Serum (FBS, 10270-106), Antibiotic-Antimycotic (A/B, 15240-062) was purchased from Invitrogen (Germany). (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) MTS reagent was purchased from Promega (UK).

V.2.2. κ-Carrageenan hydrogel preparation

An aqueous solution was prepared by dissolving the κ-carrageenan powder (herein referred as κC) in distilled water and heating at 60 °C while stirring constantly from 30 min until complete and homogeneous dispersion of the material was observed. The 1.5 % (w/v) hydrogel solution was sterilized by steam power during 30 minutes at 120 °C. The samples were produced in the form of discs using cylinder moulds and a complete gelation was achieved with KCl immersion for 10-15 minutes at room temperature in order to stabilize the 3-dimensional structure. Afterwards, the gels were washed with PBS to remove the excess of ions. Water content, degradation rate and cell culture experiments were all performed using hydrogels discs with Ø 5±0.01 mm x 2.5±0.46 mm height.

V.2.3. Cells isolation and expansion

Human adipose derived stem cells (hASCs) were obtained from liposuction procedures, under protocols established with Plastic Surgery Dept- Hospital da Prelada, Portugal and isolated by enzymatic digestion as previously described [22]. Briefly, the adipose tissue samples were digested with 0.2 % collagenase type II (C6885, Sigma Aldrich, Germany) in PBS for 45 min at 37 °C under gentle stirring. The digested tissue was filtered with a 100 μm filter mesh (Sigma Aldrich, Germany) centrifuged at 1200 rpm for 10 min at 20 °C and the cell suspension solution washed 5 min with lysis buffer to remove the erythrocytes. The adherent hASCs were expanded in Minimum Essential Medium (MEM) alpha Medium (12000-063, Invitrogen USA) with 10% (v/v) FBS, 1% antibiotic-antimycotic and sodium bicarbonate with media changes every three days. Nasal cartilage was harvested from the nasal septum of adult patients undergoing reconstructive surgery under a protocol established with Hospital de S. Marcos, Braga, Portugal. The chondrocytes isolation procedure followed a protocol presented elsewhere [23]. Briefly, the human nasal septum cartilage tissue was cut into pieces, washed in sterile PBS solution and incubated in 20 mL of trypsin-EDTA solution for 30 min at 37 °C on agitation. Then, trypsin was removed and 20 mL of filter sterilized collagenase type II solution (2 mg/mL) in basic medium was added, and the mixture incubated for 12 h. The digested tissue and cell suspension solution was centrifuged at 200g for 7 min and the supernatant removed. The cell pellet

was washed with lysis buffer, centrifuged several times and finally resuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium - high glucose (DMEM, 5671, Sigma), containing 10 mM HEPES (H403475, Sigma) buffer pH 7.4, 1% antibiotic-antimycotic, 20 mM L-alanyl glutamine (G8541, Sigma), 1x MEM non-essential amino acids (MEM NEAA, 11140-035, Sigma) and 10% (v/v) FBS supplemented with 10 ng/ml basic fibroblast growth factor (bFGF, AF100-18B, PeproTech, UK). ATDC5 (cell line mouse 129 teratocarcinoma AT805 derived, ECACC, UK) cells were expanded in Ham's F-12 cell culture medium (D-MEM/F-12 (1:1), 42400-028 Gibco, UK) supplemented with 10% FBS (Gibco, UK), 2 mM L-glutamine (G854, Sigma), and 1% antibiotic solution, until obtaining the necessary number of cells for the experiments.

V.2.4. Hydrogel characterization

V.2.4.1. *WATER CONTENT AND DEGRADATION RATE*

Hydrogel samples (111.15 mg ± 12.66) prepared according to the procedure described above and accurately weighed (w_s) were incubated for 7, 14 and 21 days in fresh culture medium and PBS buffer. Simultaneously, samples of hydrogels loaded with hASCs, hNCs and ATDC5 (prepared as described in the section bellow) were immersed in specific culture medium for each type of cell and incubated at 37 ^{0}C for the same time periods. At the pre-determined time intervals, the medium/PBS was removed from the samples, the hydrogels were lyophilized and the dry weights were measured (w_d). The water content of hydrogels was calculated from the equation $(w_s - w_d)/w_s \times 100$. The degradation rate was defined as the time needed for the gel to degrade and for calculation we considered the equation 1. The medium was replaced twice a week and the studies were performed in triplicates.

$$Degradation\ rate\ (\%) = \frac{Final\ weight}{Initial\ weight} \times 100 \qquad (1)$$

V.2.5. Cell encapsulation into κ-carrageenan hydrogels

The κ-carrageenan hydrogels were produced using the ionotropic gelation method described above and cells, namely ATDC5, hNCs and hASCs, were encapsulated at a density of 5×10^6 cells/ml and cultured for 7, 14 and 21 days. The mixture composed of the κ-carrageenan aqueous solution and the cells suspension was resuspended until complete homogenization. Hydrogel samples loaded with different cell types were prepared using sterile polystyrene cylinder moulds. Discs with encapsulated hASCs were cultured in chondrogenic differentiation medium composed of Dulbecco's Modified Eagle's

Medium-low glucose (D5523-DMEM Sigma, USA), supplemented with 1% Antibiotic-Antimycotic, ITS+1 Liquid Media Supplement (I2521- insulin-transferrin-selenium - liquid media supplement, Sigma, USA), 17mM L-ascorbic acid (A4544 Sigma, USA), 0.1M sodium pyruvate (P4562 Sigma, USA), 35mM L-proline (P5607 Sigma, USA), 1mM dexamethasone and 10ng/ml of Human TGF-β1(Transforming Growth Factor-β1, 14-8348, eBioscience). Encapsulated hNCs and ATDC5 were cultured in the previous mentioned culture mediums. Additional controls consisted of κ-carrageenan hydrogel samples without cells, kept in the same culturing conditions for the selected time periods. At the end of each time of culture the samples were retrieved, washed with PBS solution and further processed according to the characterization assays to be performed, as described below.

V.2.6. Biological evaluation

V.2.6.1. *Cells morphology and viability (calcein AM assay)*

The morphology of the different cells, before encapsulation was examined using an inverted light microscope (Zeiss, Axiovert 40 CFL, 459306) and images were obtained using a digital photo camera (PowerShot G6, Canon). The viability of the cells encapsulated in the κ-carrageenan hydrogel along the culture period was assessed using the Calcein AM assay. For this purpose, Calcein AM dye (C3099, Invitrogen, SUA) solution of 1/1000 was prepared in culture medium. At the end of each time point of the study, the κ-carrageenan hydrogels samples with encapsulated cells were collected from the culturing plates and incubated in the calcein AM solution for 15–30 min at 37 °C and afterward washed in sterile PBS. The stained samples were placed on a microscope slide and observed under a fluorescent microscopy with a 485±10 nm optical filter (Reflected/Transmitted light Microscope, Zeiss Germany).

V.2.6.2. *Metabolic activity: MTS assay*

The metabolic activity of hASCs, hNCs and ATDC5 cells encapsulated in κ-carrageenan hydrogel and cultured for the pre-determined time points was assessed using the CellTiter 96 One AQueous One Solution Cell Proliferation Assay kit (MTS, Promega, Madison), that is based on bioreduction of the substrate, into a brown formazan product by dehydrogenase enzymes in metabolically active cells and is commonly used for cell viability evaluation. Briefly, cell–hydrogel constructs (*n=9*) were washed in PBS and placed in a mixture containing serum-free cell culture medium DMEM (without phenol red) and MTS reagent at a 5:1 ratio and incubated for 3 h at 37 °C in a humidified atmosphere containing 5 %CO2, at the end of which 100 μL was transferred to 96 well plates and the OD determined at 490 nm.

V.2.6.3. *Cell proliferation: DNA Quantification*

The proliferation of the encapsulated cells in the κ-carrageenan hydrogels was assessed using a fluorimetric double-strand DNA quantification kit (P7589-PicoGreen, Molecular Probes, Invitrogen, UK). For this purpose, samples collected at 0, 7, 14 and 21 days of culturing, were properly washed in PBS and then transferred into 1.5 mL microtubes containing 1 mL of ultrapure water. Prior to dsDNA quantification, cell-hydrogel constructs and the sample controls (hydrogel samples without cells), were thawed and sonicated for 15 min. Samples and standards (ranging from 0 to 2 μg· mL^{-1}) were prepared and mixed with a PicoGreen solution in a 200:1 ratio and were added to a 96-well opaque white plate. Each sample or standard was made in triplicate. The procedure followed can be found elsewhere [24] and was based on manufacture instructions. The plate was incubated for 10 min in the dark, and fluorescence was measured on a microplate reader (Bio-Tek, Synergie HT, USA) with an excitation of 485/20 nm and an emission of 528/20 nm. A standard curve was created, and sample DNA values were read from the standard graph.

V.2.6.4. *Typical proteoglycans staining - Alcian blue, Safranin O*

Alcian blue and Safranin O staining were used to evaluate cartilage extracellular matrix component deposition, namely glycosaminoglycans, on samples that were collected at the end of the experiment (21 days of culture). Alcian blue (Sigma A3157) staining was performed by incubating the monolayer cells or the cell laden hydrogels for 30 minutes. After that, the stain was poured off and the samples were washed and dehydrated. The Safranin O staining consisted of staining the cells with Weigert's iron hematoxylin working solution for 7 minutes and 0.1 % Safranin O for 5 minutes. Samples were washed after each staining step, left to air dry and then rinsed in absolute alcohol. Stained cells were observed under a light microscope (Reflected/Transmitted light Microscope, Zeiss Germany) and images captured using a camera (Axion MRc5, Zeiss).

V.2.6.5. *RNA isolation and quantitative real-time RT-PCR analysis of chondrogenic markers*

The mRNA expression of chondrogenic genes (Sox9, aggrecan, collagen type I, collagen type II and collagen X) by the different cell types studied encapsulated in k-carragenan hydrogels was quantified by real time qRT-PCR analysis. Total RNA was extracted using TRI Reagent® RNA Isolation Reagent (T9424, Sigma, USA) following the manufacture's instruction. First-strand complementary DNA (cDNA)

was synthesized from 2 µg of RNA of each sample reverse transcribed with qScript™ cDNA Synthesis Kit (Quanta Biosciences, USA) in a 40 µL reaction using a MJ Mini™ Personal Thermal Cycler (Bio-Rad Laboratories, USA) machine. Real-Time qRT-PCR was performed to detect amplification variations using PerfeCTa® SYBR® Green FastMix®, (Quanta Biosciences, USA) on Eppendorf Mastercycler® ep realplex gradient S machine according to manufacturer's instructions in a 20 µL reaction contain 2.5 µL of each primer. The relative quantification of the gene expression was calculated using the $2^{-\Delta_{Ct}}$ and $2^{-\Delta\Delta_{Ct}}$ method [25]. The mRNA expression levels of target genes were normalized to the average expression of endogenous housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) value. Each quantitative real time PCR run was carried out with an initial incubation at 95 °C for 2 min followed by 45 cycles of denaturation (95 °C, 30 s), corresponding annealing temperature for 30 s and extension step at 72 °C for 30 s. A melting curve of 21 minutes and a hold step at 5 °C was performed at the end.

V.2.7. Statistical analysis

Sets of triplicates were expressed in mean ± standard deviation for water content and degradation rate (n=3), MTS, DNA (n=9) and qRT-PCR data (n=3). First, a Shapiro-Wilk test was used to ascertain about the data normality. The results indicated that at the 0.05 level, the data was significantly drawn from a normally distributed population and analysis of variance (ANOVA one way) followed by Tukey test was used to determine significant differences between groups and conditions. The difference was considered significant when $p < 0.05$ [26].

V.3. Results and Discussion

Tissue engineering is an emerging approach for the regeneration of damaged cartilage tissue due to disease or trauma [27] and since cartilage lacks self regenerative ability it is essential to develop methodologies for delivery of clinically relevant number of cells and/or cytokines and growth factors to the defect site. It is well known that in many situations, cells alone are not able to efficiently initiate a regeneration process once implanted, due to the dispersion or immune system rejection. Cell encapsulations techniques through hydrogels systems confer means to overcome some of these problems [28]. The motivation of using a κ-carrageenan hydrogel as a cell support was to take advantage of its intrinsic properties and composition, being a hydrophilic polysaccharide with high swelling properties, characterized by its sulphate group's content, similar to GAGs constituents. Additionally, it is a thermo/reversible and an ionic gel with the ability to form hydrogel at conditions that

allow *in situ* gelation and direct encapsulation of cells. These hydrogels have been investigated as drug delivery or growth factor delivery systems [29-31] immobilization of enzyme [32] but have been very little explored until the present time for cartilage restoration [33]. In this study we aimed at analyzing the behavior of κ-carrageenan hydrogels as encapsulation systems for adipose stem cells and primary chondrocytes.

V.3.1. Water content and degradation rate

Besides the increase in the mass of the hydrogels, high water content ability may influence the biological behavior of the cells. The matrix environment, in which cells are grown, influences the type and extent of cellular response, which in turn affects cell viability and proliferation. Therefore the water content/ degradation rate of the developed hydrogels was evaluated in simulated physiological conditions, as the diffusion, exchange of nutrients and waste throughout the entire hydrogel are related to the level of water being substituted with the surrounding medium. κ-carrageenan has an abundant number of hydrophilic groups, such as sulphate groups, which can promote a high water uptake in the structure. No previous dehydration of the hydrogels was performed, envisioning a better simulation of their application at the in *vivo* scenario. Significantly higher water content was observed for the hydrogels immersed in PBS as compared to the hydrogels dipped in DMEM/10 %FBS (Figure A1), after 21 days, possibly due to the ionic composition of the solutions. Previous research studies have shown that the swelling behavior of hydrogels depends of external environment such as ionic strength, temperature, pH [34]. The high water content of the hydrogels immersed in PBS could relate to the higher exchange between the ions from the medium and the potassium ions needed for the cross linking of κ-carrageenan hydrogel. Thus, the lower water content registered for the hydrogels exposed to DMEM/10 %FBS can be explained by the lower ionic exchange but also due to possible binding of the fetal bovine serum proteins, present in the culture medium, to the κ-carrageenan hydrogels sites. Nevertheless, all the hydrogels samples exhibited water contents superior to 98% either in DMEM/10 %FBS or in PBS medium. The degradation experiments were performed at neutral pH, resembling physiological conditions, (Figure 1, A2) and, in general, the results show a higher decrease in dry mass for hydrogels in PBS as compared to hydrogels incubated in DMEM/10 %FBS, in agreement with the water uptake results. Water content and degradation of the hydrogels with encapsulated cells, were also evaluated after culturing for 7, 14 and 21 days at 37 ^{0}C (Figure 1, B1 and B2). The highest water uptake was registered for the κ-carrageenan laden with ATDC5 cells, followed by hydrogels loaded with hNCs and hASCs cells. The mass loss difference found between the different cells type could be

associated to the particular stage of their growth cycle at each time point analyzed. For hASCs, this behavior could be linked to a higher extracellular matrix formation, since the stem cells undergo differentiation stimulated by the culturing media. In fact, cells encapsulation and extracellular matrix deposition may result in progressive increase in the mass or in the mechanical properties for the hydrogels, as shown before for other systems [35]. In this study, this was corroborated with the qRT-PCR data (see below) as we observe an increase in the aggrecan and collagen gene expression for hASCs and hNCs (demonstrating higher ECM deposition) and the lowest expression for ATDC5 cells.

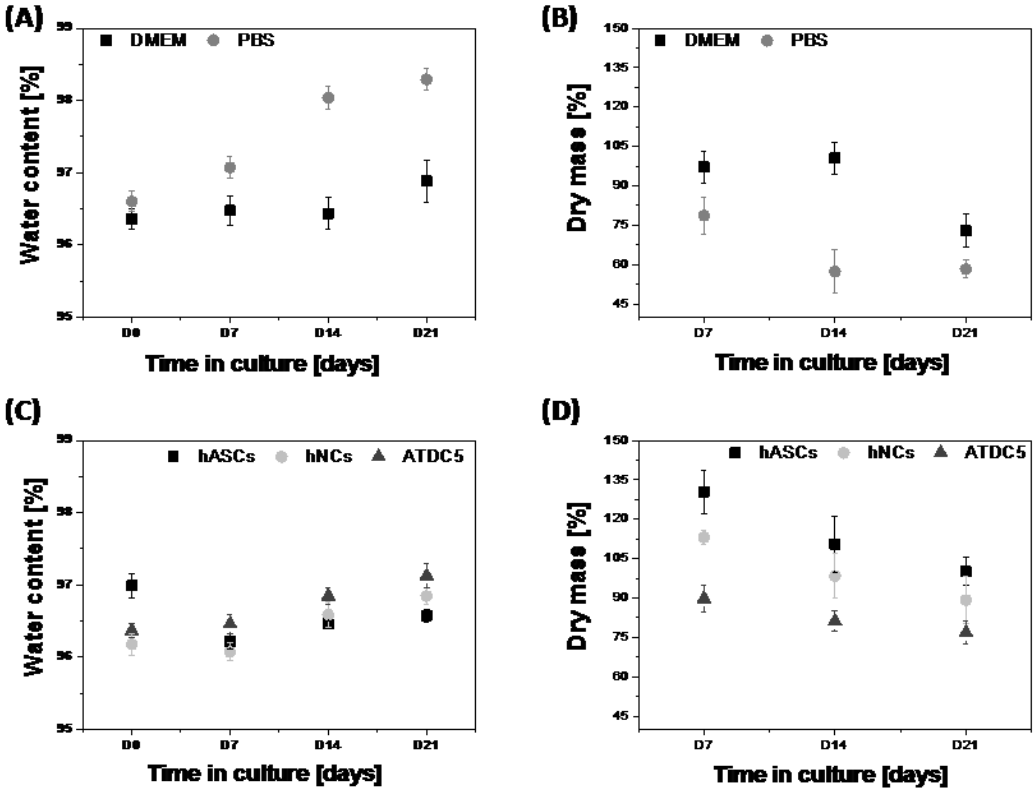

Figure V- 1. (A) Water content and (B) degradation rate of κC hydrogels as a function of time, registered after immersion in DMEM/10%FBS and in PBS solution at 37 °C and pH 7.4. (C) Water content and (D) degradation rate of κC hydrogels with encapsulated cells (hASCs, hNCs, and ATDC5) versus immersion time in culture medium (specific for each type of cell). Values reported as averages ($n = 3$) ± SD.

V.3.2. Cell morphology and distribution

Adult stem cells have recently received great attention as an alternative resource to overcome the limited supply, as well as the dedifferentiation process often observed in primary chondrocytes *in vitro* expanded in monolayer. The use of cell lines in cell based therapies is not considered safe as these

cells are often derived from tumors and typically accumulated genetic changes with increasing passage levels [36]. Optical microscopy was used to analyze hASCs, hNCs and ATDC5 cell morphology in monolayer after 7 days in culture (Figure 2A). Images depict the morphology of the different cells types, hASCs and hNCs presenting fibroblast like appearance due to chondrogenic phenotype instability and ATDC5 cells exhibiting extended, polygonal shape [37]. In monolayer culture, ATDC5 cell seemed to achieve confluence faster than hASCs and hNCs. Once different cells were encapsulated in the same hydrogel, the cell behavior was expected to be different, independently of additional influence of the hydrogel matrix [38]. Fluorescence microscopy images of the cell laden hydrogels are presented in Figure 2B. The viability of encapsulated cells within the polysaccharide hydrogel was confirmed by the positive cell staining with Calcein AM. The fluorescence images also show homogeneous distribution of all cells encapsulated in the hydrogels, while hASCs depicted a tendency to form cell clusters.

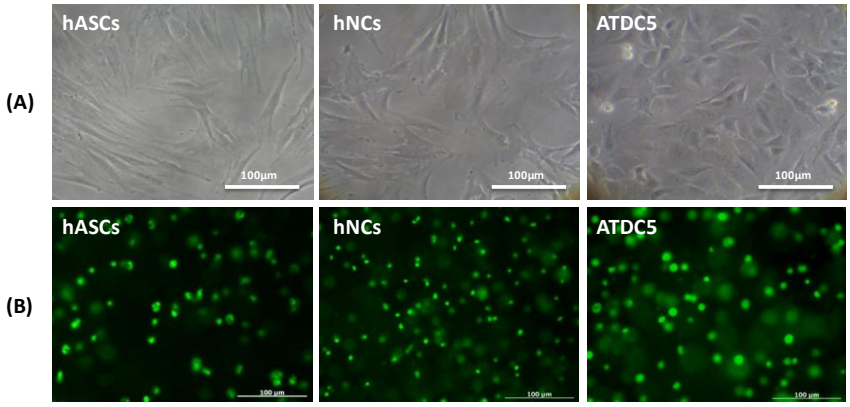

Figure V- 2. (A) Micrograph images obtained from 2D-cultured cell monolayer of hASCs, hNCs, and chondrogenic cell line after 7 days in expansion; magnification 20× with 100 µm scale bar. (B) Calcein AM staining of hASCs, hNCs, and ATDC5 encapsulated in κC hydrogels on day 7 of culture; magnification 20× with 100 µm scale bar.

V.3.3. Metabolic activity and proliferation of encapsulated cells

Viability of encapsulated cells within κ-carrageenan hydrogels during 3 weeks of culture was assessed using the MTS assay. In general, it was observed a decreased viability over time for all types of cells encapsulated in κ-carrageenan hydrogel (Figure 3A), as it is typically observed in cells encapsulated in hydrogels [39-41]. This drop in cell proliferation could be due, at least partially, to the fact that cells are being released from the hydrogels systems during culturing owing to degradation of the hydrogels. Furthermore, there are works reporting that ions needed for the hydrogel formation may influence the cell viability/proliferation and that low concentration have higher cell viability [42, 43]. Thus, remaining

potassium ions could have also contributed to the decrease in cells viability. The effect of hydrogel concentration and thus its stiffness is also thought to affect encapsulated cells, since it is known the inverse dependency between the permeability and the stiffness [44]. The data obtained also demonstrated that human adipose derived stem cells and nasal chondrocytes, once encapsulated within the biodegradable hydrogel, are more metabolically active than ATDC5 cells ($p < 0.05$). DNA quantification (Figure 3B) shows that hASCs encapsulated in κ-carrageenan hydrogels exhibit higher cell content than hydrogels laden with hNCs and ATDC5. This difference was found statistically significant ($p < 0.05$) at 14 and 21 days of culture. There are several hypotheses for the observed differences among the cells used. Each cell type was incubated in a different medium therefore we cannot rule out the effect of the cell culture medium and added supplements. This is probably related to the different growth profile of the different cells analyzed. Cell metabolic activity and proliferation data clearly indicates that κ-carrageenan, an easy-to-handle, simple and non toxic hydrogel, formed under mild conditions with *in situ* gelation properties, sustains cell growth and proliferation for long time in culture using different cell types.

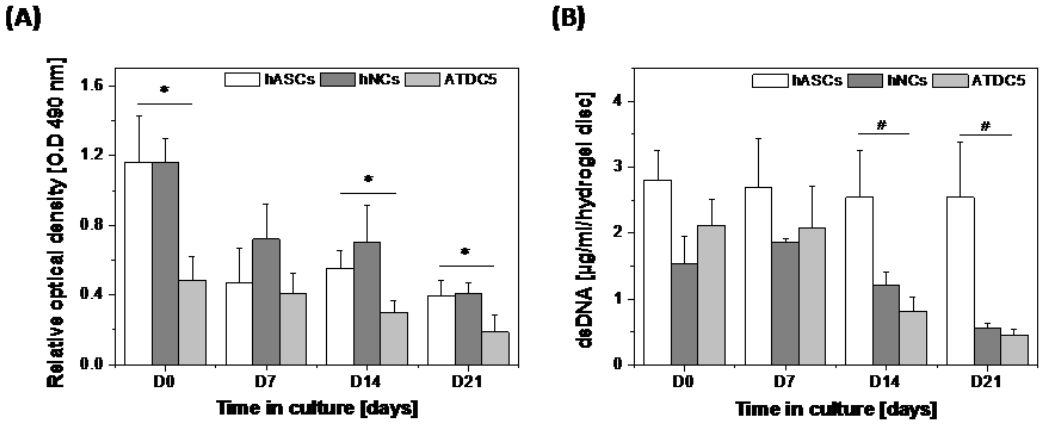

Figure V- 3. (A) Metabolic activity results based on MTS test performed on 0, 7, 14, and 21 days of culture. (B) Cell content obtained from by DNA quantification of hASCs, hNCs, and ATDC5 encapsulated in κC hydrogels and cultured for 21 days in specific mediums. Error bars represent means ± SD (n = 9). The statistics using one-way ANOVA indicates that the mean difference is significant at the 0.05 level ($P < 0.05$). The symbol ∗ indicates statistical significance between hASCs or hNCs and ATDC5 cell type in terms of metabolic activity response, and # implies statistical significance on day 14 and 21 between hASCs and hNCs or ATDC5 in the proliferation rate.

V.3.4. GAGs deposition

The glycosaminoglycans (GAGs) deposition in the hydrogels laden with the different cell types was evaluated by histological staining. Light microscopy images of sample sections obtained from hydrogels with encapsulated hASCs, hNC or ATDC5 cells, stained with Safranin O and Alcian blue, revealed positive staining for both cartilage ECM markers, confirming the extracellular matrix secretion by all the cell types embedded in the κ-carrageenan hydrogel, after 3 weeks of culture (Figure 4). It is known that the extracellular matrix is not a static structure but a dynamic network of molecules secreted by cells [45, 46] and thus the staining indicated a progressive increase in intensity over the period of culture (data not presented). The cell concentration chosen for the cells experiments was $5x10^6$ cells/ml, based on previous works [17, 47], and was the same for all the cell types investigated to allow a more straightforward comparison among the outcomes produced by the different cell types. Nevertheless, cell density has an important role in extracellular formation and may affect differently the different cell types used herein [48]. It was noteworthy that the distribution of the cartilage matrix in the hydrogels was homogeneous for all cells types. Furthermore, adipose derived stem cells exhibited stronger staining intensity, clearly demonstrating the chondrogenic differentiation undergone by these cells embedded in k-carragenan and stimulated by the chondrogenic supplements in the culture media. The round shape cell morphology observed is a further indication of chondrocyte phenotype achieved by hASCs [49]. These results show that κ-carrageenan hydrogels is not only acting as a cell vehicle, but is also supporting cells functionality, namely the chondrogenic differentiation, suggesting that this material can actually mimic ECM properties to a certain range, rendering them attractive for cartilage regeneration strategies.

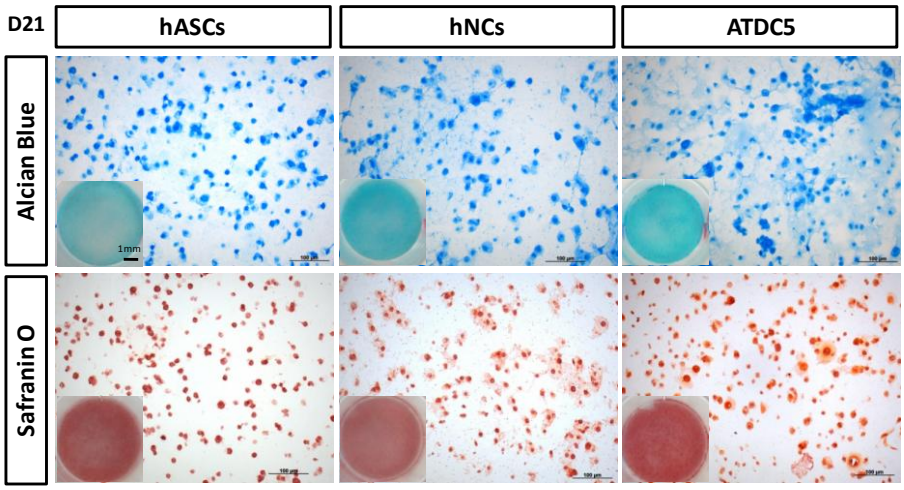

Figure V- 4. Optical microscopic images of histological sections obtained from κC hydrogels with encapsulated cells, collected after 21 days of culture and stained with Alcian Blue and Safranin O. The

scale bar corresponds to 100 μm and the magnification used was 20×. Embedded pictures correspond to light microscopy images of stained hASCs, hNCs, and ATDC5 cell monolayers after culturing for 3 weeks.

V.3.5. Real-time qRT-PCR

The results obtained from the genotypic quantification of cartilage related genes for hASCs, hNCs and ATDC5 after 7, 14, and 21 days of culture within κ-carrageenan hydrogels are presented in Figure 5. The relative expression of the cartilage specific genes was normalized against the housekeeping gene *GAPDH* and compared to initial culture time. A constitutive expression of all mRNA transcripts (i.e. *SOX9*, aggrecan – *AGCR*, collagen type I - *COL1*, collagen type II - *COL2* and collagen type II - *COLX*) were found during the 21 days of culture. In general, hASCs encapsulated in κC showed higher levels of all markers analyzed, except *collagen X*, as compared to hNCs and ATDC5 cells. Specifically, the mRNA levels in hASCs cells for day 21 in culture were found to be upregulated for the following molecules: *SOX9* (39.72-fold; $p < 0.05$), *AGCR* (53.57-fold; $p < 0.05$), *type I collagen* (10.35-fold; $p < 0.05$), *type II collagen* (94.23-fold; $p < 0.05$) and *type X collagen* (5.7-fold; $p < 0.05$). *SOX9* followed a particular high expression pattern, increasing until the last day (Figure 5A). It has been demonstrated that a highly expressed *SOX9* stimulates the production of the *AGCR* and *COL2* [50]. In fact, the results obtained from statistical analysis of the *AGCR* expression by hASCs showed significant increase ($p < 0.05$, Figure 5B) between 14 and 21 days of culture. Along with aggrecan [51], collagen type-II, is a major structural component of cartilage, particularly articular cartilage [52]. Collagen type II is the most important protein produced by chondrocytes and the tensile property of cartilage is depended of that, while aggrecan is responsible for the compressive properties [53]. The expression of the *COL2* transcript was detected for all time points, with low values for day 14 and a significant increase for day 21 ($p < 0.05$, Figure 5D). Even if *collagen type 2* exhibits a decreases expression at day 14, *aggrecan* gene expression has an increase, as these chondrogenic markers are interplaying together being expressed differently during the chondrogenic differentiation. In contrast, for hASCs, collagen type I expression showed no significant increases between 14 days and 21 days of culture (Figure 5C) suggesting that it was formed an elastic cartilage-like tissue (characterized by high expression of collagen type II) instead of fibrocartilage, usually characterized by a the expression of collagen type I. In this system, hASCs were likely stimulated down the chondrogenic pathway by TGF-β1 growth factor present in the chondrogenic medium [54]. Significant increase mRNA levels of *COL2* gene was registered for hNCs during the 21 days of culture ($p < 0.05$) although at lower levels as compared with

the fold increase expression for the stem cells (Figure 5D). For the ATDC5 cells it was registered a high expression for *COL2* gene and a low upregulation for *AGCR* at day 14 but no significant levels of the remaining genes analyzed were recorded (Figure 5D and 5B). Interactions with κC matrix may have subsequently guided cell behavior favoring chondrogenic induction, thus explaining the regulation in *COL2* gene expression among different cell types, as suggested by other works [55]. *Collagen type X* is expressed in identical levels values for all types of cultures and all time point (Figure 5E). All cell types show an increased expression marker of this gene, frequently associated with the dedifferentiation of chondrocytes, suggesting that increasing culturing time may compromise the functionality of these cells [56, 57]. ATDC5 culture produces chondrocytes maturation and apoptosis, indicating that this cell line is also suitable to study *in vitro* the mineralization process [58]. In summary, the data gathered from real time RT-PCR analysis demonstrated that hASCs and nasal chondrocytes lead to better results in terms of chondrogenic potential when compared to ATDC5 cells. Moreover, hASCs showed higher phenotypic and functional characteristics against hNCs, demonstrating that these cells could be an alternative to the autologous approach already used in clinical applications. The obtained results also indicate that κ-carrageenan hydrogels are able to stimulate specific cellular responses at the molecular level when the encapsulated cells present a different growth profile with respect to early and late cellular events in the growth profile of cells.

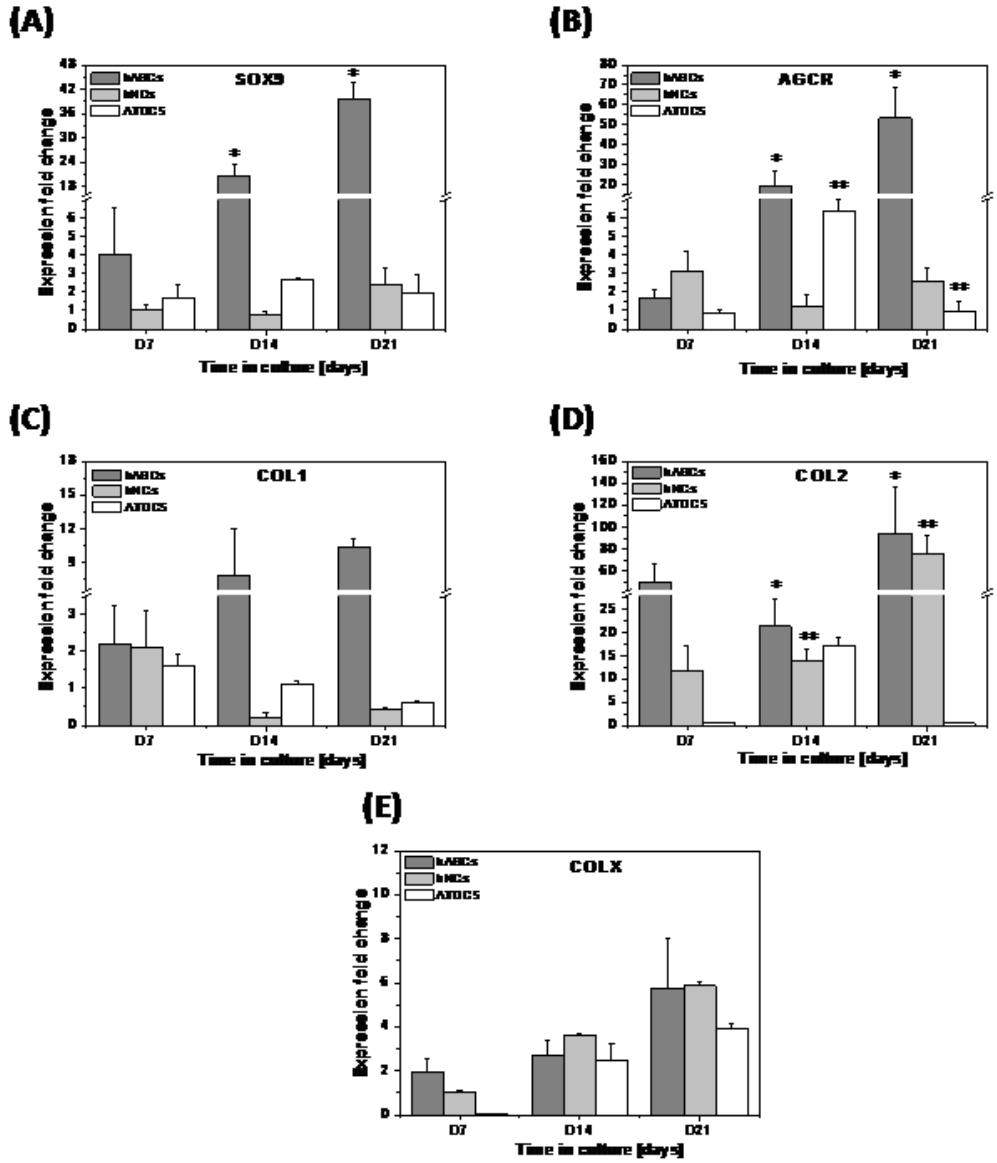

Figure V- 5. Real-time analysis of chondrogenic-specific gene expressions—namely, (A) *SOX9*, (B) *aggrecan*, (C) *collagen type I*, (D) *collagen type II*, and (E) *collagen type X*—based on the mRNA produced by the encapsulated cells after 7, 14, and 21 days of culture. The expression of these genes was normalized against the housekeeping gene *GAPDH* and calculated by the Livak method. Error bars represent means ± SD. Statistical analyses were conducted using one-way ANOVA for n = 3; $P < 0.05$.

V.4. Conclusion

The κ-carrageenan systems enabled the viability and proliferation of different cells during long term cell culture and showed its efficiency to support the production of an organized extracellular matrix and other chondrogenic features, as well as its ability to support the chondrogenic differentiation of human adipose stem cells. In fact, hASCs showed a better cellular response when encapsulated in κ-

carrageenan hydrogels than primary chondrocytes obtained from nasal septum cartilage. Taking into account these finding together with their wider availability and easier harvesting, it is possible to conclude that hASCs could serve as an alternative cells source to the chondrocytes transplantation therapy. Altogether, the results obtained from this study clearly indicate the great potential for the application of k-carragenan laden with hASCs in cartilage regeneration.

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Chapter VI. CRYOPRESERVATION OF CELL LADEN NATURAL ORIGIN HYDROGELS FOR CARTILAGE REGENERATION STRATEGIES

Chapter VI*

CRYOPRESERVATION OF CELL LADEN NATURAL ORIGIN HYDROGELS FOR CARTILAGE REGENERATION STRATEGIES

ABSTRACT

The time span needed for obtaining a functional cartilage substitute using tissue engineering strategies, together with the need for specific patient oriented constructs has stimulated the growing interest for developing "off-the shelf" products. One way to deliver such products is based on long-term storage processes, such as cryopreservation, that will provide clinical substitute available as needed and could be adapted to an autologous immediate solution for the patient. The aim of this study was to examine the effects of cryopreservation on the chondrogenic differentiation characteristics of human mesenchymal derived stem cells isolated from adipose tissue and encapsulated in κ-carrageenan hydrogels. These bioengineered constructs are anticipated to participate in a cartilage regeneration strategy providing temporary habitation for cell survival, proliferation and production of extracellular matrix which is expected to replace the hydrogel, enhancing the regeneration of native tissues in clinical settings. The results obtained show that the hydrogels withstand the cryopreservation with dimethyl sulfoxide, maintaining their structural integrity, while assisting cells proliferation and chondrogenic potential after cryopreservation. Thus, cell encapsulation systems of natural based hydrogels seem to be an interesting approach for the preservation of cartilage tissue engineered products.

Keywords: Cryopreservation, natural origin polymers, hydrogels, cell encapsulation, κ-carrageenan.

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VI.1. INTRODUCTION

One of the main prospects of cartilage tissue engineering is the possibility of developing custom-made solutions for regenerative medicine on an individual patient basis, a major challenge being the preservation and storage of the bioengineered constructs aimed at ready-to-use applications. Since the process of developing tissue engineered constructs is typically time demanding, approaches to facilitate and accelerate their clinical use have emerged. Assuming this, the cryopreservation of cells, as well as more complex systems such as cell encapsulation devices, tissue-engineered constructs, and even laboratory-produced tissues and organs may be critical in the future. Thus, the scientific increment in the literature and current clinical experience for the repair of injured or diseased articular cartilage supports the need for providing such novel products like cultured cells on engineered tissues constructs to improve the clinical market options.

Cryopreservation of various mammalian cell types has been a standard procedure for many years [1-3] envisioning cell preservation for future outcomes. Cell encapsulation is one of the primary techniques to colonize tissue-engineered hydrogels as cells are immunoprotected from the host environment once implanted. The most applied cryopreservation protocols use dimethyl sulfoxide (DMSO) (1 to 20% v/v) in serum-supplemented culture medium combined with slow-freezing rates [4]. However, the cryopreservation of tissue engineered constructs is typically associated with difficulties in preserving the 3D structural properties of the constructs, and the challenge stands for reducing to minimum the cryo-injury, predominantly related to ice formation upon freezing and thawing [5]. Nevertheless, complex cell-scaffolds constructs kept under routine cryopreservation procedures are emerging as potential banking constructs in regenerative medicine applications [6, 7]. Alternatives have been proposed with the use of several cryoprotectants [8-10], vitrification solutions [11, 12] or the entrapment of cells on artificial extracellular matrix (ECM), like hydrogels [13-16]. Artificial ECM/hydrogels may be viable to protect the cells from outside damage, whereas the cryoprotectants will protect the cell from intracellular or intercellular ice crystals formation. This system allows cells to be protected against mechanical damages during ice crystallization and the danger of disrupting cell–cell interactions is reduced through immobilization [17, 18].

Natural hydrogels have been widely used in the biomedical field, as cell encapsulation and delivery systems, envisioning their application in several regenerative medicine therapies [19-22]. Among the different polymers studied, κ-carrageenan is a very versatile, thermosensitive hydrogel used to develop ionotropic matrices for *in situ* immobilization of cells [23]. Carrageenan belongs to a family of linear, water soluble, sulfated, anionic polysaccharides extracted from marine red algae [24, 25]. They are

highly flexible molecules with the ability to shape into different formats at room temperature due to its thermosensitive characteristic. The formation of irreversible carrageenan hydrogels can be obtained in the presence of potassium ions, which leads to the formation of more stable hydrogels. More recently, κ-carrageenan based systems have been proposed for cartilage regeneration strategies [26, 27] and for delivery systems of proteins and growth factors by our group [28-30].

Although the cryopreservation of cells or tissues is a common method that provides advantages for cellular therapies [31] aimed at bioengineering cartilage, few studies have addressed the impact of cryopreserving constructs as a combined cell therapy. A fundamental objective of this work was to verify the viability, proliferative and differentiation capacity of human adipose derived stem cells (hASCs) into chondrocyte-like cells while encapsulated in the κ-carrageenan hydrogels, demonstrating the ability to maintain the metabolic functionality after cryopreservation. Another crucial goal towards the applicability of the proposed system into clinical settings was the analysis of the effect of cryopreservation on the morphological and mechanical properties of the system as a functional construct. The outcomes of this study suggest that this strategy could provide a ready-to-use pool of cells for cartilage clinical treatments.

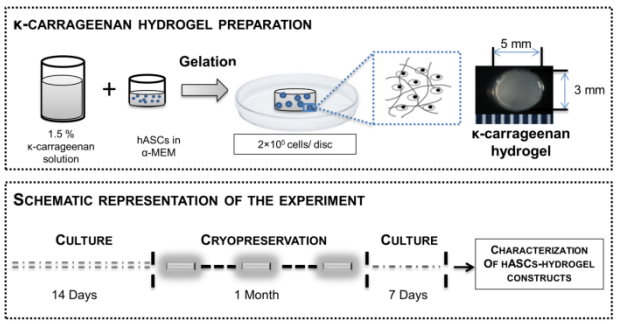

Figure VI- 1. Study design showing details of the preparation and cryopreservation process for the developed hASCs-κCR construct.

VI.2. Materials and Methods

VI.2.1. Preparation of ionic κ-carrageenan hydrogels

The ionic gelation process of the natural polysaccharide based κ-carrageenan (κCR, 22048, Sigma) was performed as described elsewhere [32]. Briefly, the κCR powder was mixed with distilled water to obtain the 1.5 % (w/v) polymeric concentration. The solution was warmed up in a water bath at 50 °C, stirring until the complete dissolution and afterwards sterilized (121 °C for 30 min) before cell culture

studies. In order to form hydrogel samples in the form of discs, 5 mL of κCR solution was poured into sterile plastic Petri dishes of 55 mm ∅ and the freshly formed hydrogels were punched out into disc shape sample of 5 mm ∅ x 3 mm height. The hydrogel formation was further stabilized by cross linking with 5 % (w/v) potassium chloride (KCl, P5405, Sigma) for 15 minutes. Afterwards the hydrogels samples were washed with PBS to remove the excess of KCl present on the surface.

VI.2.2. hASCs isolation and expansion

Human subcutaneous fat tissue samples were obtained under protocols established with the Plastic Surgery Department of Hospital da Prelada (Porto, Portugal). Adipose tissue derived stem cells from four female donors with a mean age of 35.25 years (± 8.55) and BMI of 26.26 (± 2.74) were isolated by enzymatic digestion as previously described [33]. Briefly, the adipose tissue samples were digested with 0.075 % collagenase type II (C6885, Sigma) in phosphate buffer saline (DPBS, 21600-044, Invitrogen) for 45 min at 37 °C under gentle stirring. The digested tissue was filtered with a 100 μm filter mesh, and centrifuged at 1000 g for 10 min at 5 °C. Afterwards the cell suspension was washed for 10 min with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH= 7.3) to remove the erythrocytes, and additionally centrifuged at 800 g for 10 min at 5 °C. The adherent hASCs were expanded in basal medium composed of Minimum Essential Medium alpha (MEM, 12000-063, Invitrogen) with 10% (v/v) fetal bovine serum (FBS, 10270, Invitrogen), 1% (v/v) antibiotic-antimycotic solution (15240-062, Gibco®), and 2.2 mg/mL sodium bicarbonate ($NaHCO_3$, S5761, Sigma) with medium changes every three to four days. Cells were subcultured at a cell density of 3.5 x 10^3 cells/cm^2 until achieving a sufficient cell number to be used in the experimental assay. The hASCs used in this study were at passage three.

VI.2.3. Cells encapsulation

The κCR aqueous solution and the cell suspension were mixed until complete homogenization, and hydrogel discs samples loaded with hASCs were formed as described above, using sterile moulds. The hASCs were encapsulated at a density of 5 × 10^6 cells/cm^3 into the κCR hydrogels and cultured for 14 days either in chondrogenic or basal medium. The chondrogenic induction medium was composed of Dulbecco's Modified Eagle's Medium-low glucose (DMEM, D5523, Sigma), supplemented with 3.8 mg/mL $NAHCO_3$, 1 % (v/v) Antibiotic-Antimycotic solution, 10 % (v/v) FBS (fetal bovine serum, SH3007103, Fisher Scientific), 1× ITS+1 (insulin-transferrin-selenium - liquid media supplement, I2521, Sigma), 17 mM L-ascorbic acid (A4544, Sigma), 0.1 M sodium pyruvate (P4562, Sigma), 35

mM L-proline (P5607, Sigma), 1 mM dexamethasone (D2915, Sigma) and 10 ng/mL of human transforming growth factor-β1 (TGF-β1, 14-8348, eBioscience). κCR hydrogel samples without cells kept in culture for the same time points under the same cell maintenance conditions and cell pellets, were used as controls. For the preparation of the 3D micromass pellets, aliquots of 2×10^5 cells in 0.3 mL of medium were centrifuged at 1500 rpm for 15 min. Pellets were cultured for 14 days in a 37 °C humidified atmosphere with 5 % CO_2, and medium was changed once a week. At the end of cell culturing, part of the samples were retrieved, rinsed in PBS solution and characterized by light microscopy, mechanical analysis, live dead-assay, DNA quantification assay, histology analysis and reverse-transcription polymerase chain reaction (RT-PCR) assessment. Simultaneously, the remaining part of the samples was removed from culture and cryopreserved (Fig. 1).

VI.2.4. Cryopreservation process

Samples that underwent cryopreservation were frozen for one month before being cultured for additional 7 days in BM or CM. The cryopreservation process was based on the following protocol: each sample was individually placed in a polypropylene cryovial (479-0821, VWR) containing a solution of 10 % (v/v) dimethyl sulphoxide (CryoSure DMSO, 11-32-30216, Wak-Chemie Medical GMBH) in FBS. The samples were cooled down to 4 °C using an ice bath (30 minutes). Afterwards, cryovials were moved into a conventional freezer (-20 °C; 1-2h) and then moved into a -80 °C freezer. After 12 hours at -80 °C, samples were stored inside a liquid nitrogen tank (Statebourne Biosystem) at -196 °C for one month.

In total the experiment set-up included five groups, consisting of: i) hASCs- κCR hydrogels construct cultured in chondrogenic medium (CM); ii) hASCs-hydrogels construct cultured in basal medium (BM); iii) κCR hydrogels cultured in basal medium; iv) pellets cultured in chondrogenic medium (CM) and v) pellets cultured in basal medium (BM). Samples were evaluated before (BC corresponds to 14 days in culture before cryopreservation) and after cryopreservation (AC corresponds to 7 days of culture after cryopreservation). All samples were subjected to light microscopy observation, DMA (dynamic mechanical analysis) assays, live dead-assay, DNA quantification assay, histology and RT-PCR analysis (Table 1).

Table VI- 1. Summary of the tested group cell-hydrogel constructs and pellets cultured in either basal medium (BM) or chondrogenic medium (CM) and characterization techniques performed on samples retrieved before (AC) and after cryopreservation (BC).

Groups	Culture condition			Light microscopy	DMA	Live-dead assay	dsDNA	Histology				RT-PCR
								TB	H&E	Coll II	Coll I	
Hydrogels Tested	i)	CM	BC	3	20	3	24	12	12	12	12	18
	ii)	BM	AC	3	20	3	24	12	12	12	12	18
	iii)	κCR		3	20	nd	24	nd	nd	nd	nd	-
Pellets Control	iv)	CM	BC	-	nd	nd	24	-	-	-	-	-
	v)	BM	AC	-	nd	nd	24	-	-	-	-	-

CM: chondrogenic medium; BM: basic medium; κCR: κappa-carrageenan hydrogel; BC: before cryopreservation; AC: after cryopreservation; - data not showed; DMA: dynamic mechanical analysis; nd: not determined; dsDNA: double strand DNA; TB: toluidine Blue; H&E: haematoxylin and eosin; Coll II: collagen type II; Coll I: collagen type I; RT-PCR: reverse transcription polymerase chain reaction.

VI.2.4.1. *MORPHOLOGY AND MECHANICAL PROPERTIES*

The full structure and morphological characteristics of the developed discs with encapsulated cells (cultured in CM or BM) and without cells (cultured only in BM) both before (BC) and after cryopreservation (AC) were observed under an inverted light microscope (Zeiss, Axiovert 40 PG-HITEC) equipped with a digital camera (Axiocam MRc5, Zeiss). The mechanical properties of 1.5 % (w/v) ionic κCR hydrogel discs BC and AC were characterized by DMA (TRITEC Admin 8000B, Triton Technology). The viscoelastic measurements were carried out at 37 °C in 5% KCl and the samples were immersed in the culture medium until performing the assay. After equilibration, the DMA spectra were obtained during a frequency scan in the range of 0.1–10 Hz. The experiments were performed under constant strain amplitude (50 μm). A small preload was applied to each sample to ensure that the entire disc surface was in contact with the compression plates before testing and the distance between plates was equal for all samples being tested. At least five samples were tested for each condition.

VI.2.4.2. *VIABILITY AND PROLIFERATION OF THE ENCAPSULATED HUMAN ADIPOSE DERIVED STEM CELLS*

Live and dead assays using fluorescence labeling were used to assess the viability of the cells. In short, samples were collected, placed in a 48-well plate, and washed with PBS. Subsequently, 500 μL of 1 mg/mL Calcein AM in PBS (1:500, C3099, Invitrogen) was added to each sample for 10 min at 37 °C and later washed with sterile PBS. Afterwards, the samples were incubated with 300 μL of 1.5 mM propidium iodide in PBS (PI; 1:1000, P1304MP, Invitrogen) for 5 min at room temperature, previous to microscopic analysis (Reflected/Transmitted light Microscope, Axioimager Z1M, Zeiss).

DNA quantification was performed using the fluorimetric PicoGreen double-stranded DNA assay according to the manufacturer instructions (Quant-iT™ PicoGreen® dsDNA Kit, P7589, Invitrogen). Briefly, cell-hydrogel constructs, collected at each time period, were rinsed twice in PBS to completely

remove the culture medium, placed in a microtube containing 1 mL ultra-pure water, and stored at - 80 °C to induce the lysis of cells by osmotic and thermal shock, respectively. Then, samples were thawed and the supernatant collected for the dsDNA quantification assay. The fluorescence of the samples was measured with a microplate reader (Synergie HT, Izasa) at Ex 480 nm and Em 520 nm. The DNA concentration was calculated using a standard curve (DNA concentration ranging from 0-2 μg mL^{-1}) relating to the amount of dsDNA to the fluorescence intensity.

VI.2.4.3. *Histological evaluation - Typical proteoglycans and H&E staining*

Samples collected before and after cryopreservation were fixed with 3.7 % (v/v) neutral buffered formalin solution and stored at 4 °C until analysis. Samples were further processed by a series of dehydration steps, embedded in paraffin (Microm EC350-2, Thermo Scientific) and sectioned at 3 μm (Microm HM 355, Thermo scientific). Toluidine Blue (TB) staining was used to evaluate the deposition of glycosaminoglycans (GAGs), a cartilage ECM. TB (T3260, Sigma) coloration was performed by dipping the cell laden hydrogels sections for 2–3 s in the staining solution. Afterwards, the stain was poured off and the samples washed, dehydrated, cleared in xylene (1.08681.1000, VWR), and mounted for further analysis. For haematoxylin and eosin (H&E) staining, after hydration, sample sections were colored with Papanicolaou Harris hematoxylin (05-12011/L, Bio-optica) for 3 minutes, washed in running tap water, and afterwards a blue stain enhancement was performed by an immersion in 0.5 % ammonium hydroxide solution (05002, Sigma) for 5–10 seconds. The sections were washed in running tap water and stained in Eosin-Y (05-M10003, Bio-optica) for 30 seconds. Finally, slides were dehydrated through series of alcohol immersions from 30 until 100 % (v/v) alcohol. The stained cells were visualized under a light microscope and images were taken by a digital camera (Axion MRc5, Zeiss).

VI.2.4.4. *Immunolocalization of type I and II collagens*

Fixed samples with 3.7 % (v/v) formalin solution were paraffin embedded and sectioned. Before removing the paraffin, the slides were warmed, and the antigen retrieval was performed for 20 minutes at 95 °C using 10 mM citrate buffer. Afterwards the sections were washed in PBS, and endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide (31642, Sigma) for 5-15 min. Sections were washed with PBS and blocked with 2.5% horse serum from R.T.U. Vectastain® Universal Elite ABC Kit (PK-7200, Vector Laboratories) for 1 h to avoid nonspecific staining and further incubated with primary antibodies (mouse anti-collagen II antibody - MAB1330, Millipore, and rabbit anti-collagen I

antibody - ab292, Abcam) overnight at 4 °C, in a humidified atmosphere. Afterwards the slides were incubated with secondary antibody (R.T.U. Vectastain® Universal Elite ABC Kit) for 1 h at room temperature and developed with DAB substrate kit for peroxidase (SK-4100, Vector Laboratories). Slides were counterstained with haematoxylin, mounted and visualized under the light microscope. Controls were performed using normal horse serum replacing the primary antibodies.

VI.2.4.5. *RNA ISOLATION AND GENE EXPRESSION ANALYSIS (RT-PCR)*

RT-PCR analysis was performed to detect the gene expression of typical markers for chondrogenic differentiation, namely *aggrecan*, *collagen type II*, *Sox9*, *collagen type X* and *collagen type I*. Total RNA was extracted using TRI Reagent® RNA Isolation Reagent (T9424, Sigma) following the manufacture's instruction. First-strand complementary DNA was synthesized from 2 μg of RNA of each sample reverse transcribed (qScript™ cDNA Synthesis Kit, Quanta Biosciences) in a 20 μL reaction using an Eppendorf Mastercycler® ep realplex gradient S machine. mRNA expression of the genes of interest was measured in the hASCs laden κCR hydrogels as well as in the pellet culture systems, both before and after cryopreservation. The transcript expressions of target genes were analyzed to the expression of endogenous housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). The primer sequence was obtained from Primer3 software (v 0.4.0), and synthesized by MWG Biotech (see Table 2). Conventional PCR was performed using a High- Fidelity DNA Polymerase (Finnzymes) and dNTP Mix (Finnzymes) as well as a primer concentration of 0.2 mM in a 35 cycle 3-step reaction. The PCR products were detected in a 1.3 % agarose gel (SeaKem®LE agarose, Lonza) using a 100 bp DNA ladder (GeneRuler™ SM024, Fermentas). Images from the gels were taken using a UV Transiluminator (BioSpectrum AC Chemi HR 410, UVP) (n=3).

Table VI- 2. Primers used for RT-PCR analysis and expected sizes of the PCR products.

Target gene	Ascension No.	Primer Forward	Primer Reverse	Amplicons	Tm [°C]
Aggrecan	NM_001135	5'TGA GTC CTC AAG CCT CCT GT	5'CAG TGG CCC TGG TAC TTG TT	171 bp	60.4
Collagen II	NM_033150	5'GGG AGT AAT GCA AGG ACC AA	5'ATC ATC ACC AGG CTT TCC AG	175 bp	57.4
Sox9	NM_000346	5'TAC GAC TAC ACC GAC CAC CA	5'CTC CTC AAG GTC GAG TGA GC	217 bp	56.2
Collagen X	NM_000493	5'CAG GCA TAA AAG GCC CAC TA	5'AGG ACT TCC GTA GCC TGG TT	179 bp	57.4
Collagen I	NM_000089	5' AGC CAG CAG ATC GAG AAC AT	5'ACA CAG GTC TCA CCG GTT TC	109 bp	55.3
GAPDH	NM_002046	5'TGC ACC ACC AAC TGC TTA GC	5'GGC ATG GAC TGT GGT CAT GAG	87 bp	60.6

bp - base pairs; GAPDH - glyceraldehyde-3-phosphate dehydrogenase

VI.2.5. Statistical analysis

All data values are presented as mean ± standard deviation. Differences were determined to be statistically significant with $p < 0.05$. For statistic analysis one-way ANOVA analysis of variance between groups with Tukey post test was carried out.

VI.3. RESULTS

VI.3.1. Cell laden hydrogel morphology before and after the cryopreservation process

In the present study we have analyzed the effect of cryopreservation process on the chondrogenic features developed by the hASCs after encapsulation in κCR sulfated hydrogels. Cell laden hydrogels were cultured for 2 weeks before being cryopreserved for one month under standard conditions. Afterwards, constructs were thawed and cultured for an additional 7 days period.

In order to analyze the morphology of hydrogels and the encapsulated cells, micrographs images were obtained from hASCs-hydrogel constructs culture in CM and BM, after and before cryopreservation (Fig. 2). Macroscopically, the integrity of the hydrogels did not seem to be affected by the cryopreservation/thawing process, considering the maintenance of the shape after cryopreservation, with minor variations in the hydrogel volume (Fig. 2 insets). Exceptions in terms of surface morphology were found in the control hydrogel (without cell) where it is possible to observe shrinkage of the cryopreserved sample and wrinkles on the surface. In cell laden hydrogels, it is noticeable a better spreading of the hASCs before cryopreservation compared to after cryopreservation, when cell appeared more rounded with less visible cell to cell connections. Even so, microscopic images suggest that the cellular density is maintained after cryopreservation and that the hydrogel stability is overall maintained, keeping its structural cylindrical shape and the cells inside the hydrogels. Nevertheless, κCR hydrogels are sensitive to ionic environments, naturally present in cell culture media. Thus, despite κCR hydrogels show a perfect shape immediately after cryopreservation, the materials seem to be less stable after 7 days in culture, yet without compromising their functionality as cellular laden system.

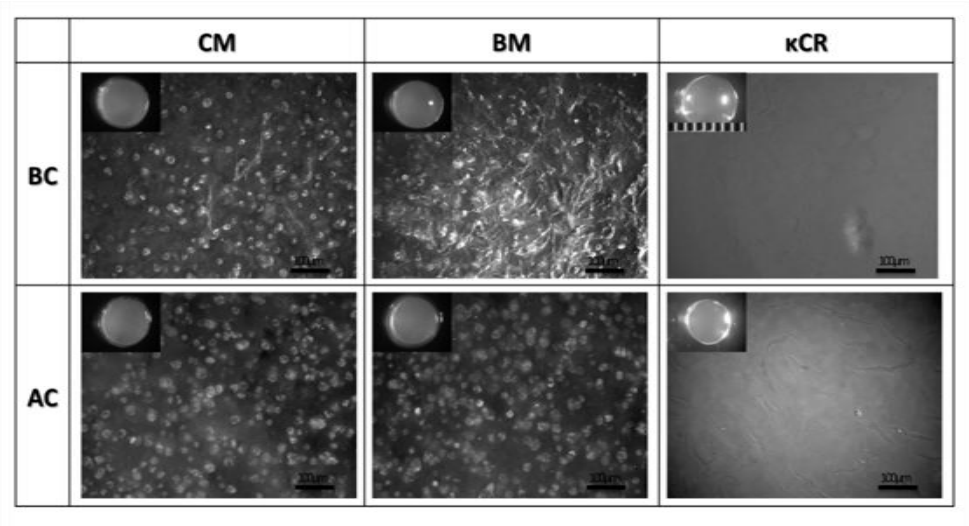

Figure VI- 2. Optical microscopy images of hASCs-hydrogel construct and κCR hydrogel without cells collected before and after cryopreservation. The insets represent the full image of the discs, whose details are showed in the magnified image. Scale bars represent 100 μm.

VI.3.2. Mechanical properties

In the present study, it was performed a preliminary assessment of the stability of these hydrogels upon cryopreservation by dynamic mechanical analysis. DMA allowed determining the mechanical properties of κCR hydrogels with encapsulated hASCs after different culturing times (14 days before cryopreservation and 7 days after cryopreservation) in either chondrogenic or basal medium (as compared to κCR hydrogels without cells). The analysis was performed with the hydrogels immersed in a KCL solution at 37 °C and throughout a physiological relevant range of frequencies. Storage (elastic) and loss (viscous) components of the complex modulus were determined and are presented in Fig. 3. The elastic modulus (E') curve shows the viscoelastic behavior of κCR hydrogels with or without cells before and after cryopreservation and how the stiffness of the polymeric material changes with the frequency. Increase in E' is observed for the conditions which were not exposed to the freeze-thawing cycles as compared to the samples that were cryopreserved and are not frequencies dependent. The storage modulus for the hASCs-hydrogel exposed to chondrogenic medium before cryopreservation holds the highest values, showing a stiffer structure, and tends to increase with increasing frequency from 85 to 120 kPa (Fig. 3). The influence on the loss factor (tan δ) of the freezing process and the growth factor addition to the medium is also presented in Fig. 3. All hydrogels showed a frequency-dependent behavior, with damping increase in frequencies higher than 2 Hz. In general, a more prominent increase was observed in the hydrogels exposed to the freeze-thawing cycles.

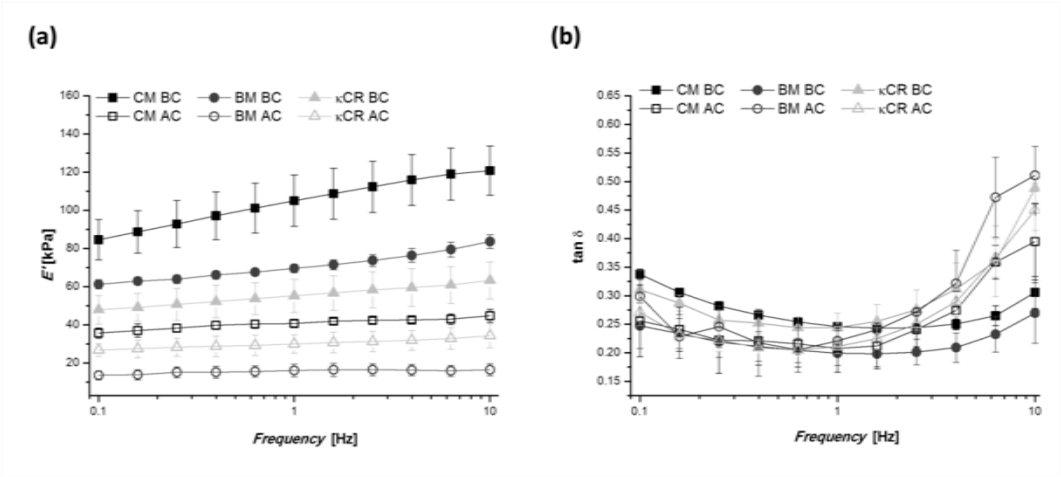

Figure VI- 3. (a) Storage modulus (E') and (b) loss angle (tan δ) obtained from dynamic mechanical analysis upon compression of hydrogels with encapsulated hASCs and plain hydrogels cultured in chondrogenic and basal medium before and after cryopreservation.

VI.3.3. Viability and proliferation of hASCs encapsulated in κ-carrageenan hydrogels

Fluorescence microscopy images provided information about cell viability and morphology and the cell content was evaluated by DNA quantification. Fig. 4a depicts the live/dead imaging of hASCs encapsulated in κCR hydrogels and cultured in chondrogenic or basal medium. Stained in green are the viable cells, which enzymatically converted the non-fluorescent cell-permeant calcein AM into the intensely fluorescent calcein. A higher hASCs density in the hydrogel constructs is observed prior to cryopreservation with a lower number of positive viable cells present after cryopreservation. In the chondrogenic formulation, cells appear to be more agglomerated and spread compared to the basal conditions for the earlier time point. After cryopreservation, cells cultured in chondrogenic medium seem to be more biologically active than the ones cultured in basal conditions.

The DNA content corroborated with the live/dead data analysis. Cell content measurements (Fig. 4 b, c and d) tend to decrease after cryopreservation. A decrease in cell content was registered ($p<0.05$) after cryopreservation both for constructs cultured in basal or chondrogenic media, but higher values ($p<0.05$) were registered for the samples cultured in chondrogenic medium both BC and AC (Fig. 4b). The cellular content of the hASCs-hydrogel construct and the pellet cultured in chondrogenic medium evidenced a similar pattern, when compared to the respective samples cultured in basal medium (Fig. 4c and 4d). Nevertheless, the cellular content decrease is less accentuated in hASCs-hydrogel constructs than in the pellets, suggesting that the hydrogels supported cell content maintenance in culture post thawing.

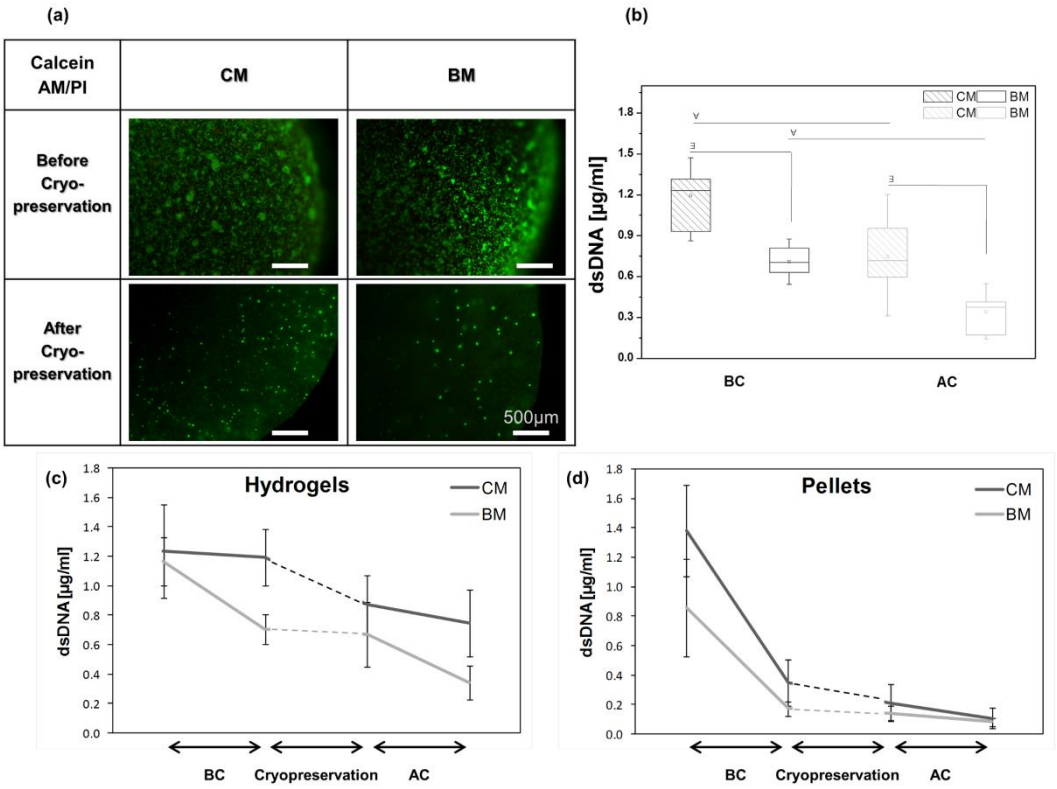

Figure VI- 4. Cell viability (a) and proliferation [b), c), d)] of hASCs-hydrogel constructs culture in either chondrogenic or basal medium, before (BC) and after (AC) cryopreservation. a) Merged green/red fluorescence images of hASCs, Calcein-AM indicating live cells (green) and Propidium iodide dead cells (red). Scale bars represent 100 µm. b) Cell content obtained from dsDNA quantification assay for chondrogenic (CM) and basal (BM) medium. Values indicate mean ± standard deviation. Significant differences in cell amount for chondrogenic or basal medium BC and AC were registered ($p<0.05$). c) and d) Cell proliferation tendency based on dsDNA quantification for the hydrogels and the pellet control group.

VI.3.4. Histological analysis

Histological evaluation provided qualitative information to detect possible changes in cell morphology, ECM production, and overall hydrogel structure before and after cyoprezervation. Fig. 5 shows the light microscopy images of the constructs stained with TB and H&E stains. H&E showed well distributed hASCs throughout the hydrogel section in chondrogenic or basal medium either before or after cryopreservation. Also, the high amount of hASCs observed indicated that cells were successfully encapsulated in the hydrogels and remained enclosed in the polymeric structure during the cryopreservation/thawing cycle, not being particularly affected by the minor hydrogel fissures caused

by cryopreservation (Fig. 5). TB staining revealed the presence of negatively charged glycosaminoglycans (GAGs) in the cross-sections of the hASC-hydrogels constructs (Fig. 5). The localization of GAGs, one of the major cartilage ECM components, indicates possible deposition of a cartilage-like ECM. ECM localization is detected before and after cryopreservation indicating that the freeze/thaw protocol didn't affect extensively the GAGs production, and consequently the intrinsic metabolism of differentiated hASCs. After cryopreservation and in chondrogenic conditions, hASCs tend to agglomerate into multiple-cell vacuole. The micrographs also evidence that cells are individually or paired gathered inside vacuole-like structures typically related to the chondrocytes morphology and to native cartilage features. When encapsulated in κCR hydrogels, hASCs adopted a more spherical morphology, which is typically observed in encapsulation systems.

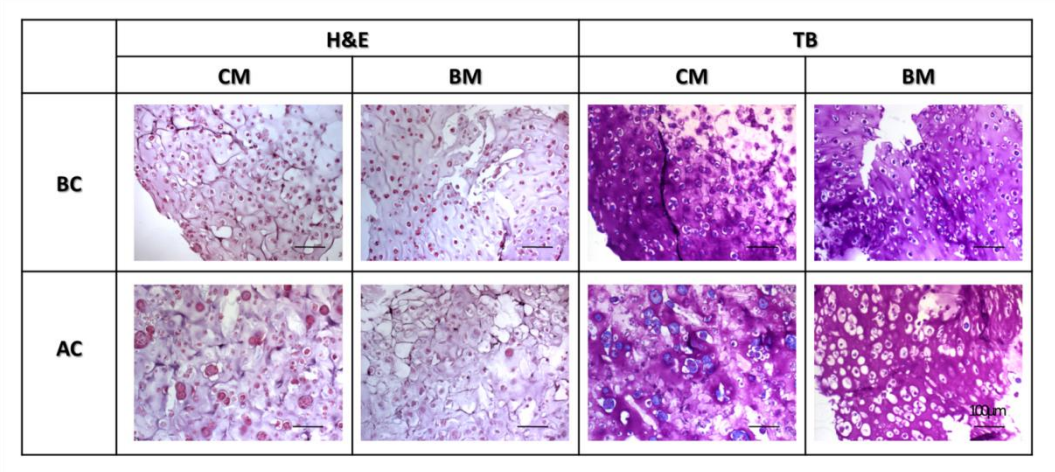

Figure VI- 5. Hematoxylin - eosin (H&E) staining for cellular detection and overall distribution within the hydrogel. Detection of cartilage extracellular matrix, namely glycosaminoglycans (by Toluidine Blue, TB) of chondrogenically induced hASCs and undifferentiated hASCs encapsulated in κCR hydrogels discs. hASCS-hydrogel constructs were collected and stained BC and AC. Scale bars represent 100 µm.

VI.3.5. Immunohistochemisty

Complementarily, immunohistochemical analysis of the biomacromolecules typically present during cartilage development was investigated. Distribution of cartilage-like matrix was evaluated through immunolocalization of cartilage matrix proteins before and after the freeze-thaw process and posterior culture for additional 7 days in chondrogenic or basal medium. Before cryopreservation, the deposition of collagen type II seems higher in the chondrogenic formulation when compared to collagen type I. Fig. 6 shows that cells present in the κCR hydrogel were positively stained for both types of collagens

and these cartilage matrix components were homogeneously distributed throughout the hydrogels. Before cryopreservation, the hASCs cultured in basal medium seems to show lower expression of collagen II and collagen I when compared to the chondrogenic samples. After cryopreservation, the expression of collagen I seems to decrease in hASCs-hydrogels cultured in basal medium, evidenced by a decrease in the brownish color revealed by DAB, and increased blue associated to hematoxylin counterstain. Moreover, it was highlighted the presence of cells in lacunae, characteristic of native articular cartilage tissue. Overall, the cells maintained their chondrogenic properties throughout the culturing time before the freeze-thawing cycle. The intensities of collagen type I immunolocalization seems to increase in the chondrogenic condition after cryopreservation. Furthermore collagen type II and collagen type I expression appear to be similar in basal medium after cryopreservation. These cartilage matrix markers were distributed throughout the hASCs-hydrogel construct. Colony formation in the medium supplemented with TGF-β1, AC is detected, as previously referred. Overall and despite minor variations, cryopreservation seems not to interfere in the expression of proteins associated to cartilage ECM.

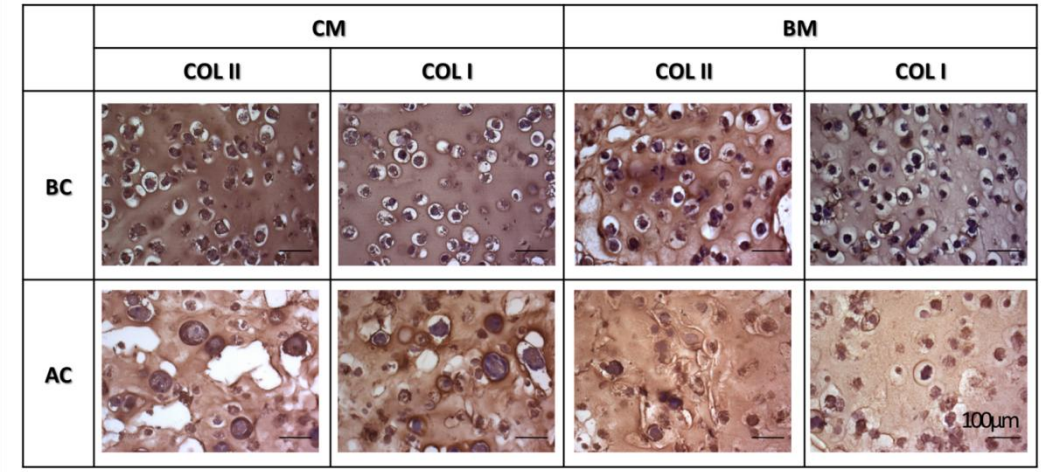

Figure VI- 6. Immunohistochemical localization of collagen type II and collagen type I proteins in hASCs-hydrogels exposed to chondrogenic or basal medium BC and AC. Scale bar = 100 µm.

VI.3.6. RT-PCR

To complement the immunostaining results, RT-PCR was performed in hASCs-hydrogel BC and AC. The results obtained from the genotypic evaluation of cartilage-related genes expressed by hASCs within κCR hydrogels are presented in Fig. 7. The expression of the cartilage specific genes was verified in an agarose gel, using *GAPDH* as housekeeping gene. The expression of transcripts associated to cartilage

(i.e. *SOX9*, *aggrecan*, *collagen type I*, *collagen type II* and *collagen type X*) was observed by 14 days of culture, just before cryopreservation. hASCs encapsulated in κCR showed higher levels of *collagen II*, *collagen X*, and *SOX9* in chondrogenic conditions. Conversely, *aggrecan* seems to be more expressed in basal medium, while *collagen I* is similarly expressed in basic or chondrogenic media. In general, after cryopreservation, gene expression seems to be lower, almost undetected, in all conditions studied, except for the housekeeping gene and *collagen X*.

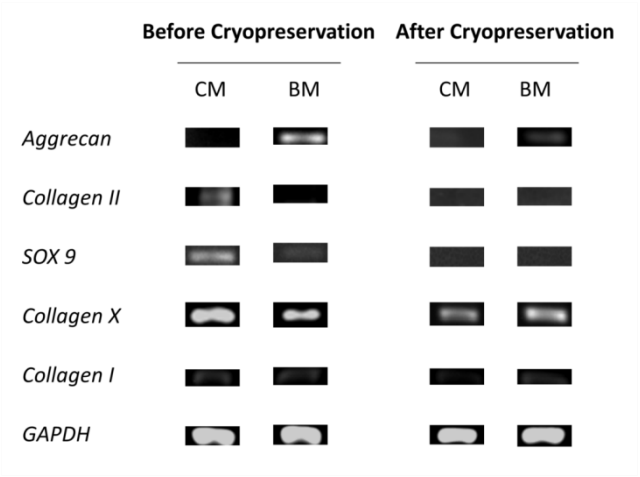

Figure VI- 7. Representative image of the electrophoresis gels reporting the expression of cartilage-related genes by RT-PCR. The gel shows the results obtained before and after cryopreservation protocol and cultured in chondrogenic or basal medium.

VI.4. Discussion

Presently, cryopreservation protocols are the only mean for long term preservation of biological samples, thus playing an important role in cell and tissue banking assuming a greater importance when tissue engineering is approaching an everyday reality [34]. The relevance in exploiting "ready-to-use" cartilage engineered constructs fulfilling patient-specific characteristics combined with adequate preservation methodologies, anticipates the need for development of "out of shelf" tissue substitutes. It is envisioned that such approaches will play a central role in future applications with tissue engineered products, as constructs could be prepared in advance, and scaled up assuring immediate availability and accessibility for clinical use.

This study investigates the influence of cryopreservation using standard protocols in a promising engineered product composed of hASCs encapsulated in κCR hydrogels. Moreover, we proposed to evaluate if the chondrogenic potential as well as cellular viability and proliferative capacity of

encapsulated human adipose derived stem cells is restored after established freeze/thaw cycles. The method selected for cryopreservation of the developed system combines the cryoprotectant DMSO with FBS, using a temperature gradient until a long term preservation in liquid nitrogen (-196 °C).

At a macroscopic level, evidence suggested that this procedure does not significantly affect hydrogel structural integrity, as the morphology and stability of hASCs- κCR hydrogels was not altered radically by the cryopreservation process. The fissures observed on the surface of the hydrogel are not interfering with the overall constitution of the hydrogel (Fig. 2). The fact that κ-carrageenan hydrogels successfully underwent cryopreservation while preserving their structural integrity indicates that these carriers are easily manipulated and suitable to be used as efficient encapsulation systems under simple cryopreservation protocols. This statement is in accordance to literature reports which evidence the good freeze/thaw stability of carrageenan based hydrogels [35].

Moreover, dynamic mechanical analysis (DMA) was performed to better characterize the mechanical/viscoelastic properties of the hydrogels in a simulated physiological environment (wet state at 37 °C). The non-cryopreserved constructs, register an increase with frequency which is not detected for the cryopreserved construct. During the days in culture prior to cryopreservation, DMA analysis indicated an increase in storage modulus and in viscoelastic properties of κCR gels with encapsulated cells suggesting an increase in the stiffness, possibly due to the extracellular matrix production. Fig. 3 shows that in all the frequency range, the storage modulus is higher for the hASCs-hydrogel construct chondrogenically committed, which corresponds to the typical stiffness effect found more in these systems due to the cellular component and high secretion of ECM. DMA data indicated that the hASCs-hydrogels show a viscoelastic behavior and maintain their mechanical properties after a freeze/thawing process. Furthermore, obtained values were described to be in the range of constructs aimed at cartilage engineering applications with a compression modulus similar to the ones reported for agarose and alginate hydrogels [36]. The values of tan δ (higher than 0.2) reveal a clear viscoelastic behavior of the hydrogels. This is an important feature in biomaterials used for tissues subjected to periodic loads, such as cartilage, since it shows the material's ability to absorb mechanical energy. The increase of the loss factor for the freeze-thawed gels, along with the stability observed for E' values with frequency show that for high frequencies these hydrogels increase their ability to dissipate energy. Nonetheless, at frequencies corresponding to human movements, such as walking or running (around 1 Hz) no significant differences in the loss factor of the BC and AC hydrogels were observed.

Previous reports about the functional application of κCR hydrogels show their applicability for *in situ* encapsulation of different types of cells, while keeping cellular proliferation and assisting the expression of cartilage extracellular matrix markers [23]. Microscopy analysis of hASC- κCR hydrogels revealed

lower cellular density and minor morphological variations in cell shape and cell-to-cell interactions after cryopreservation. In the particular case of encapsulated cells cultured in BM, morphological changes are more significant AC as they lose most of pseudopodia and their fusiform appearance into a more chondrocytic shape. This modification may be associated to cell adjustment to the freeze/thawing cycle, which is more evident in a basic culture medium without chondrogenic biochemical supplements.

Besides the morphological aspect of the cells, the effect of cryopreservation on the viability and proliferation of hASCs encapsulated in κCR hydrogels was also studied. Overall, and despite an expected decrease observed upon cryopreservation, the constructs were able to maintain the cells viability and proliferation. It is known that the freezing/thawing process interferes with the biophysical and biochemical features of the cells, resulting in alterations of their functional competency and survival [38]. As a consequence, cryopreserved cells require a recovery time after thawing for adjusting sudden and radical micro-environmental changes. The observed decrease in cell content post cryopreservation might also be explained by the minor fissures observed in the κCR hydrogels post cryopreservation (Fig. 2), which could led to the release of some encapsulated cells from the system.

Immediately after thawing, hASCs viability/cell content levels were higher compared to longer time in culture and tend to decrease with time. This trend was found both for hASCs- κCR constructs and pellets. The explanation may reside in the fact that short time in culture after cryopreservation is not sufficient to allow cellular reboot. While the rationale behind the short time in culture after cryopreservation was to study the immediate availability and functionality of the cell-hydrogel construct, longer time in culture, are expected to assure the cellular recovery from the cryopreservation process as reported previously [37].

The more accentuated decrease in cell content observed in the pellets, as compared to the cell-laden hydrogels, suggests that hydrogels may act a protective barrier to intracellular ice formation or as a buffer for the cryoprotectant agent diffusion, as it has been previously reported [39], yet allowing the exchange of nutrients and oxygen. Interestingly, the cell contents registered for hydrogels cultured in chondrogenic conditions is significantly higher ($p<0.5$) than in basal medium cultures, before and after cryopreservation. The combination of κCR hydrogel and chondrogenic supplements may provide the necessary stimuli for cellular colonization despite chondrogenic differentiation.

Histological findings indicated cellular morphology changes after the cryopreservation process. ECM proteoglycans deposition was observed in the pericellular regions of most cell clusters. Although adult chondrocytes do not divide or establish cell-to-cell contacts, these cells are responsible to produce a cartilagineous dense ECM [41], thus maintaining cartilage integrity and function. This finding may be

explanatory to the fact that stem cell chondrogenically differentiated produce and secreted ECM components. The pre-existing vacuoles observed in the histological micrographs (Fig. 5) formed before the cryopreservation cycles, typical to the chondrocytes phenotype, enlarge after cryopreservation and were colonized with migrating cells, creating cell agglomeration into the free spaces. This finding may be associated to the fact that enhanced tissue deposition is observed nearby these aggregate structures. H&E control staining revealed cells entrapped in the hydrogels and a good stability of the complex cell-hydrogel system.

The immunolocalization of the proteins analyzed in this study (collagen type II and I) are known to be naturally present in cartilage ECM. Collagen type II is typically detected in elastic cartilage while collagen type I is involved in fibril formation of several skeletal tissues and expressed in fibrocartilage, a scar tissue, *in vivo*. Both forms of collagen II and collagen I were produced and retained in the form of ECM to an analogous extent before and after cryopreservation process (Fig. 6). Moreover, a positive localization was detected for the cells cultured in basal medium, i.e., in the absence of any chondrogenic inducer growth factor, suggesting that the 3D hydrogel environment was sufficient to stimulate the adipose derived stem cells to present typical cartilage-like morphology, namely vacuoles and rounded shape, as well as the synthesis of collagen II rich matrix. The deposition of extracellular matrix resulted from the 2 weeks of cell culturing prior cryopreservation. This time point was selected based on previous studies on hASCs chondrogenic differentiation [23]. Two-weeks were the shortest culturing time necessary to promote chondrogenic differentiation of hASCs with ECM production. The ECM observed in both chondrogenic and basal cultures may influence the cryopreservation effect on the hASCs- κCR constructs. Similarly to the functionality of the κCR hydrogels, the deposition of ECM may allow a controlled diffusion of the cryoprotectant into the cells located in the matrix [42].

Genes commonly studied for chondrogenic differentiation analysis include collagen type I, II and X, as well as *SOX9* and *aggrecan*. *SOX9*, a marker of early chondrogenesis, is also important in achieving a healthy cartilage mainly because SOX9 stimulates aggrecan and collagen II synthesis [43]. Although *Collagen X* has been associated to hypertrophic chondrocytes *in vivo*, it has also been described as a lineage specific marker for cartilage differentiation of mesenchymal stem cells [44]. It is well established that multipotent mesenchymal stromal cells (MSCs) undergoing chondrogenesis have a tendency to express collagens I and X *in vitro* [45]. Overall, the genes associated to the chondrogenic differentiation process are increased in hASCs-hydrogel constructs before cryopreservation for both culture conditions (CM and BM). After cryopreservation, gene expressions seem to be reduced in all conditions except for the housekeeping gene and for *collagen X* in chondrogenic medium. The decrease in gene expression after cryopreservation has been described in the literature [40], and may be

associated to the fact that cells are still recovering from the cryopreservation process and are still adapting to the new conditions. Although some cellular responses were already restored 7 days after cryopreservation, it is expected that later in time, cells will fully recover and express the chondrogenic related genes into the levels prior to cryopreservation [46]. Nevertheless, and since we are envisioning an "off-the-shelf" bioengineered product with immediate availability and ready-to-use application, a time compromise must be reached. That is, after cryopreservation protocols, the cell- κCR system must return to cell culturing just time enough to be functional, fulfilling the aims it was developed for.

The broad range of biological properties of MSCs attracted the interest of many researchers to be used for diverse therapeutic applications. One advantage of using MSCs in this system rely on the fact that these cells possess a high plasticity and are dynamic in terms of cellular metabolic machinery [47], reacting more promptly to physicochemical variations in the environment than primary chondrocytes, which tend to dedifferentiate when cultured *in vitro* [48]. Furthermore, the applicability of stem cells in cryopreservation systems can disclose an even more promising approach of these cryopreserved systems into the regenerative medicine field, as stem cells are immune privileged and these products may fit different patients [49].

VI.5. Conclusions

This study evidences that complex tissue engineered products aimed at clinical needs can be stored and preserved through the cryopreservation process. In the proposed system, it was observed that κ-carrageenan hydrogels pledged structural stability at a macro-scale and integrity with or without encapsulated hASCs, registering suitable mechanical properties after the cryopreservation/thawing process. Also, encapsulated hASCs maintained cell viability and chondrogenic features upon cryopreservation by restoring cellular metabolic functionalities 1 week of cell culturing after thawing the constructs. The natural polymer κ-carrageenan hydrogel seem to be resilient to the cryopreservation method used, and does not negatively interfere with hASCs chondrogenic potential demonstrating its promising application as cell carrier agent in “off-the-shelf” engineered tissue products. We envision future studies applying these systems for transplantation following an *in vivo* cartilage regeneration strategy.

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Chapter VII. EVALUATION OF THE IN VITRO AND IN VIVO BIOCOMPATIBILITY OF CARRAGEENAN BASED HYDROGELS

Chapter VII*

Evaluation of the *in vitro* and *in vivo* biocompatibility of carrageenan based hydrogels

ABSTRACT

Carrageenans are highly sulphated galactans, well-known for their thermogelation properties which have been extensively exploited in food and cosmetics industry but poorly explored in the biomedicine field. However, these natural origin polymers present a variety of properties and characteristics that may render them high potential for a number of different possible applications in this field. In this study we have assessed the *in vitro* and *in vivo* biocompatibility of κ-carrageenan hydrogels that have been explored for regenerative medicine and tissue engineering applications. For this purpose, it was evaluated first the *in vitro* cytotoxicity of the materials using a L929 mouse fibroblast cell line and next it was assessed the effect of κ-carrageenan hydrogels on the activation of human polymorphonuclear neutrophils cells (hPMNs) by the quantification of reactive oxygen species (ROS) by chemiluminescence. Subsequently it was studied the inflammatory/immune reaction to κ-carrageenan hydrogels upon subcutaneous implantation in rats. Explants were retrieved after 1 and 2 weeks of implantation, for histological and RT-PCR analysis. The cytotoxicity screening revealed that κ-carrageenan hydrogels did not significantly affect L929 metabolic activity. Moreover, hPMNs contact with κ-carrageenan resulted in a reduced and a neglectable signal regarding the detection of superoxide and hydroxyl anions, respectively. The results from the *in vivo* experiments indicated that κ-carrageenan seems to induce a low inflammatory response. Overall, the data obtained suggests that κ-carrageenan hydrogels are biocompatible and thus can be further studied for their use in target biomedical applications.

Keywords: Hydrogels, κ-carrageenan, adipose derived stem cells, chondrogenic differentiation, cartilage tissue engineering.

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VII.1. INTRODUCTION

Regenerative medicine and tissue engineering are interdisciplinary areas of research focused on generating therapies to repair, augment, or replace wounded, unhealthy or metabolically deficient tissues or organs [1-3]. In order to develop innovative and successful approaches that can provide better outcomes in specific clinical applications, it is critical to develop new biomaterials that can interface perfectly with the surrounding tissues at a structural, mechanical and specially at a biological level, once implanted or injected into the body [4, 5]. The first and main requirement for such biomaterials concerns its biocompatibility, i.e. its ability to perform in a specific situation with an appropriate host response [6]. Biocompatibility is not solely dependent on the material intrinsic characteristics but can also be defined by the target application in the sense that the material is required to induce a specific cell/tissue reaction [7]. To have a full picture of the biocompatibility of any proposed system in the biomedical field, the *in vitro* biological behavior must be fully characterized, previously to *in vivo* assays.

Hydrogels have been widely used in regenerative medicine/tissue engineering approaches due to their high water content, soft and rubbery consistency and due to their structural and functional similarities to the natural extracellular matrix of human tissues [8, 9]. Moreover natural hydrogels offer the advantage of being similar to macromolecules that the biological environment is prepared to recognize [10, 11]. The issues of toxicity and lack of recognition by cells, which are frequently provoked by many synthetic polymers, may thereby be suppressed or minimized using natural hydrogels [12]. Carrageenans are important hydrophilic polysaccharides, which consist in a family of linear and sulfated galactans used in several industrial, environmental and commercial applications as gelling, thickening, emulsifying and stabilizing agents [13]. Besides the general advantageous features of the natural origin hydrogels above described, the characteristics and thus the medical potential of these seaweed polysaccharides, has increasingly attracted the attention of researchers in recent years. Carrageenan has been used to develop controlled release systems of drug for ophthalmic/buccal preparations [14-16], and of growth factors [17-19], and for immobilization of enzymes [20]. Nevertheless, the potential of this polysaccharide has only started recently to be explored for tissue engineering and regenerative medicine purposes [21-23].

The main aim of this work was to evaluate κ-carrageenan hydrogels as potential biomaterials for the development of new tissue engineering and regenerative medicine therapies. The biocompatibility of κ-carrageenan hydrogel was firstly screened using standard *in vitro* cytotoxicity assays and then assessed evaluating the activation of human polymorphonuclear neutrophils (hPMNs) through the detection of reactive oxygen species (ROS). For the *in vivo* evaluation, κ-carrageenan hydrogels were subcutaneous

implanted in rats for histological reverse transcription polymerase chain reaction (RT-PCR) analysis. In both *in vitro* and *in vivo* experiments, agarose gels were used as control material, given their well known biocompatibility and wide use in the biomedical field [24, 25].

The outcomes of the present study demonstrated that the κ-carrageenan hydrogels did not elicit any biological negative effects, inducing an expected host response, and thus this material can be considered in subsequent studies for regenerative medicine applications.

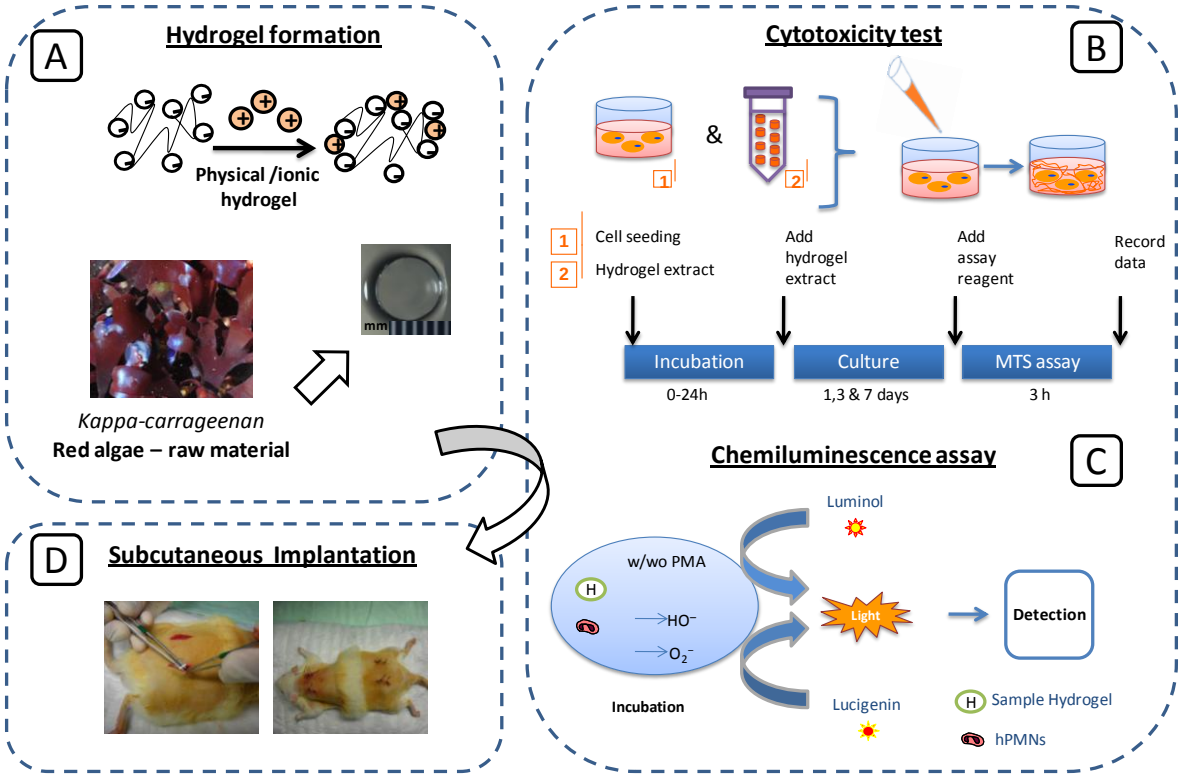

Figure VII- 1. Schematics of study design: A) raw material and hydrogel formation; B) typical protocol for cytotoxicity assays; C) overview of ROS detection assay assembly; D) implantation of the hydrogels in the created subcutaneous pockets and appearance of the animal after suturing.

VII.2. Material and Methods

VII.2.1. Preparation of materials

The κ-carrageenan hydrogels (designated as κC), were prepared as follows: first the polymeric powder (22048, Sigma) was mixed with distilled water under constant stirring at 60 °C to obtain a final concentration of 2 % (w/v). The polymer solution was then casted into a mould (plastic Petri dish) of 55 mm ∅ and kept at room temperature (RT) for 2-5 minutes forming a solid gel, which was further

stabilized for 15 minutes by crosslinking with potassium chloride (KCl, P5405, Sigma). Finally the gels were washed with PBS to remove the excess of I. Discs of Ø 8 ±0.01 mm x 2.5 ±0.46 mm height were then cut using a punching device. Agarose hydrogels were used as control material (referred from now on as AG) and were produced following standard procedures detailed elsewhere [26]. Briefly, a 2 % (w/v) sterile agarose low gel temperature (800257, MP Biomedicals) solution, prepared in sterile PBS, was heated to 70 °C for 30 seconds, until complete dissolution. The solution was cooled at RT promoting gelation due to temperature- and ionic-dependent reactions, like for the κCR (Fig. 1).

VII.2.2. In vitro cytotoxicity evaluation: indirect contact test

In the scope of the *in vitro* biocompatibility assessment it were performed, indirect cytotoxicity tests using extracts of the hydrogels being studied, based on ISO/EN 10993 part 5 guidelines [27].

VII.2.2.1. *Cell line used for cytotoxicity hydrogel extracts testing*

A mouse fibroblast L929 cell line, acquired from the European Collection of Cell Cultures (ECACC), was used for the cytotoxicity tests. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM, D5523, Sigma) supplemented with 10 % fetal bovine serum and 1 % of antibiotic-antimycotic solution. Trypsin was used to detach the cells from the culture flasks before the experiments were conducted. L929 cells were seeded in 96-well cell culture plates ($4x10^3$ cells/well, 200 µl/well), in order to reach 70 % confluence after 24 h of incubation at 37 °C, in a humidified atmosphere with 5% CO_2 (Fig. 1).

VII.2.2.2. *MEM extraction Test and MTS assay*

The polymeric κCR and AG hydrogels, previously sterilized by steam power (121 degree for 20 minutes), were extracted in complete culture medium (MEM) for 24 h at constant temperature (37 °C) and agitation (60 rpm). The ratio of hydrogel to extract fluid was constant and equal to 3 cm^2/ml. Latex rubber was used as a positive control as it is known to have a strong cytotoxic effect leading to extensive cell death and lysis. The extracts of the fully hydrated hydrogels were filtered (filter pore size: 0.22 µm) and placed in contact with the monolayer of the L929 cells. For this purpose, the culture medium from the well-plates with the L929 cells monolayers was removed and an identical volume (200µl/well) of extraction fluid of study materials and control was added. Cells grown in the presence of standard culture medium were included as negative controls. Cell response was evaluated after 1, 3,

and 7 days of incubation. The MTS assay was performed to assess the possible cytotoxicity of the hydrogels, determining the metabolic activity of the cells (Fig. 1). This assay is based on the bioreduction of the substrate (3-(4, 5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (Cell Titer 96® Aqueous Solution Cell Proliferation Assay, Promega), into a brown formazan product. Briefly, after 1, 3 and 7 days of culture, the extraction fluid was removed and a mixture containing serum-free culture medium without phenol red and MTS reagent was added to each well and incubated for further 3 hours at 37 °C and 5% CO2 atmosphere. Supernatants were then transferred to new 96-well plates and the optical density (OD) was read at 490 nm in a microplate reader (Synergy HT, Bio-TeK Instruments).

VII.2.3. In vitro assessment of human polymorphonuclear cells (hPMNs) activation

VII.2.3.1. *hPMNs isolation*

hPMN cells were isolated from heparinized peripheral blood collected from healthy volunteers following a procedure detailed described elsewhere [28]. Briefly, each 10 mL of heparinized blood was poured into 10 mL of a 6% Dextran (D8906, Sigma) solution in PBS without Ca^{2+} and Mg^{2+} (Sigma). After 20 min, about 6 mL of the top layer was removed with a glass pipette and carefully added onto 4 mL of Histopaque® (1077, Sigma). After a 25 min centrifugation at 21 °C and 2400 rpm, the bottom pellet was resuspended in 5 mL of PBS without Ca^{2+} and Mg^{2+} and the cells suspension was centrifuged for 25 min at 21 °C and 2400 rpm. After this, the supernatant was discarded and 1mL of distilled water was added and the suspension was triturated three times with the glass pipette and shacked gently for 35 s, in order to lyse the erythrocytes. Cells suspension was centrifuged for 25 min at 4 °C and 2400 rpm and the cell pellet washed again with PBS without Ca^{2+} and Mg^{2+}. The cell suspension was kept at 4 °C until the assays were performed, within a maximum of 2 h.

VII.2.3.2. *Reactive oxygen species (ROS) quantification*

The production of superoxide anions and hydroxyl radicals by hPMNs after contacting with the studied hydrogels was detected by a chemiluminescence-based method (Fig. 1C). The isolated hPMNs, were used at a final concentration of 1.3×10^{6} cells/mL. The reactive oxygen species quantification assay followed a methodology previously described [29]. In brief, to each 100 µL of cells suspension alone and in contact with the hydrogels were added 8 µg/mL PMA/PBS (phorbol 12-myristate 13-acetate, P8139, Sigma) and 100 µL of 1.5 mM luminol/PBS (09253, Sigma) or 100 µL 5.4×10^{-5}M lucigenin/PBS (B49203, Sigma). Control conditions were simultaneously set without cell stimulant, the

PMA. All the plates were maintained on ice while setting the experiment. The chemiluminescence was read up to 2 hours in a microplate reader (Sinergy HT, Bio-TeK Instruments).

VII.2.4. Hydrogel subcutaneous implantation

The *in vivo* biocompatibility was evaluated upon subcutaneous implantation of hydrogels, using a total of 20 rats (12-weeks-old Wistar male rat with an average weight between 260 and 300 g), (Table 1). All animal studies were performed accordingly to the national guidelines and conducted in accordance with Portuguese legislation (Portaria n°1005/92) and international standards on animal welfare as defined by the European Communities Council Directive (86/609/EEC). Surgeries were performed under general anesthesia, intraperitoneal (IP) administration route using a combination of medetomidine (Dormitor® - 0.5mg/Kg) with ketamine (Imalgene® - 75mg/Kg). Post-surgical pain control, subcutaneous administration route, with carprofen (Rimadyl®, 2.5-5mg/Kg) was performed for each animal. κCR and AG hydrogels discs (Ø 8±0.01 mm x 2.5±0.46 mm height) were prepared as described above. Under surgical sterile conditions, two full thickness skin longitudinal incisions (about 1 cm) containing the subcutis and the panniculus carnosus (skin and smooth muscle) were performed in the dorsum of each animal (anterior and posterior incisions). Cranial and lateral oriented subcutaneous pockets were created by blunt dissection, one in each side of the incision. The hydrogel discs were inserted into these pockets (four discs of the same materials per animal), and the panniculus carnosus and the skin were carefully sutured (Fig. 1). Negative control group was set for the time periods of implantation with empty pockets (EP). As positive control group, rats were injected with lipopolysaccharides (LPS) (1μg/g rat body weight, L4641, Sigma) 24h before the collection of samples. After each predetermined implantation time period (7 and 15 days), each animal was anesthetized with isofluorane and IP injection with an overdose of pentobarbital sodium (Eutasil® from Ceva Saúde Animal).

Table VII- 1. Animal group conditions.

Groups	Conditions	Time points	Rat numbers
Experimental	*κCR*		*6*
Control Material	*AG (std)*	*Day 7*	*6*
Negative Control	*EP (-)*	*Day 15*	*6*
Positive Control	*LPS (+)*		*2*

VII.2.5. Collection of explants

At each time point, explants were retrieved from the four implantation sites of each animal together with the respective axillary and inguinal lymph nodes. Explants were either fixed in 3.7 % formalin for histological evaluation, or frozen for posterior PCR analysis.

VII.2.5.1. *HISTOLOGICAL CHARACTERIZATION*

For histological analysis, explants were subjected to standard histological tissue processing for paraffin embedding. Sections with 4 μm thickness were obtained using a microtome (HM355S, Microm, Thermo scientific) and stained with haematoxylin and eosin (05-12011/L, 05-M10003, Bio-optica) in an automatic stainer (HMS740, Microm, Thermo scientific). Immunohistochemistry was also performed on histological sections to assess presence/localization of recruited macrophages and activated lymphocytes and thus tissue response to the implants. In brief, sections were dewaxed, rehydrated and antigen retrieval was achieved by heating at 96 °C in 10 mM citrate buffer, pH=6, for 40 minutes. Sections were washed in PBS and endogenous peroxidase activity was blocked with 0.6 % hydrogen peroxide in methanol for 10 min at RT. Afterwards, permeabilization was performed using Triton™ (X-100, Sigma) for 2 minutes followed by incubation with protein block buffer for 20 min at RT. Excess serum was removed by blotting and the sections were incubated overnight at 4 °C with either CD163 antibody (ED2, MCA342GA, AbD Serotec) to detect recruited macrophages or CD25 antibody (MCA494GA, AbD Serotec) to detect activated lymphocytes. After incubation, sections were rinsed with PBS for 5 min and incubated with polyclonal swine anti-mouse, goat, rabbit IgG antibody (Dako) for 1 h at RT. Further washes in PBS were performed prior to exposure of the sections to Vectastain elite ABC reagent (PK-6200, Vector Laboratories Ltd.) for 30 minutes at RT and the substrate reaction was developed using the 3,3′-diaminobenzidine (DAB) peroxidase substrate solution (SK-4100, Vector Laboratories Ltd.). Finally sections were counterstained with haematoxylin and mounted with Entellan® (4111, Inopat). Labeled sections were observed under a Transmitted light Microscope, (Zeiss) and images acquired with an MRc5 camera and the AxioVision V.4.8 software (Zeiss).

VII.2.5.2. *PCR ANALYSIS - CONVENTIONAL END-POINT RT-PCR*

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis was used to detect at the molecular level, the expression of inflammatory markers, namely *IL-1 alpha*, *IL-4*, and *IFN- gamma*. The housekeeping gene was glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) chosen as an endogenous control for the experiments. The detailed description of the sequence of the primers used

is presented in Table 2. The explanted tissue was transferred to eppendorfs tubes with 1 mL TRI Reagent® RNA Isolation Reagent (T9424, Sigma) and stored at -80 °C until processing. The homogenization of the biologic tissue was achieved using a mortar and pestle. Total RNA was extracted from samples following the manufacturer's guidelines (TRI Reagent®). The amounts of isolated RNA from samples with $A_{260/280}$ ratio between 1.6 and 2.0 were determined using Nanodrop ND-1000 Spectrophotometer (Bonsai 06/2008 NanoDrop Technologies). A predetermined amount of RNA from each sample was reverse transcribed into cDNA using qScript™ cDNA Synthesis Kit (Quanta Biosciences) using a MJ Mini™ Personal Thermal Cycler (Bio-Rad Laboratories) machine. Amplification of the sequences of interest was performed using a GoTaq® Green Master Mix (M7112, Promega) and primers at a concentration of 10 μM in a 30 cycle 3-step reaction. The PCR products were visualized in a 1.7 % agarose gel (SeaKem ®LE agarose, Lonza) with a DNA marker pBR322 - Hae III (A5229, AppliChem). Images from the gels were taken using a UV Transilluminator (BioSpectrum AC Chemi HR 410, UVP).

Table VII- 2. Primer pair sequences and annealing temperatures used in the RT-PCR analysis

Target gene	*Sequences*		T_m [°C]	*Bp*
	Sense	*Antisense*		
IL-1α	GCAAAGCCTAGTGGAACCAG	GCAGAAGGTGCACAGTGAGA	59.4	244
IL-4	TTTTGAACCAGGTCAACACCA	GTGAGTTCAGACCGCTGACA	57.4	-
IFN-γ	GCCCTCTCTGGCTGTTACTG	CTGATGGCCTGGTTGTCTTT	59.4	221
GAPDH	GGTGATGCTGGTGCTGAGTA	GGATGCAGGGATGATGTTCT	58.4	81

IL-1 alpha: interleukin-1 alpha; *IL-4*: interleukin-4; *IFN-γ*: interferon-gamma; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; Tm: annealing temperature; Bp: base pair.

VII.2.6. Statistical analysis

Statistical analyses for the cytotoxicity results were conducted by a two-way ANOVA with Tukey's test using the GraphPad Prism statistic software. Test ascertains about the data normality and the significance value was set at $p < 0.05$. Mean values and standard deviations are reported for the cytotoxicity measurements and *in vitro* ROS quantification assay.

VII.3. RESULTS

VII.3.1. In vitro cytocompatibility assessment

The *in vitro* cytotoxicity of the hydrogel extracts was assessed as a first screening of the materials biocompatibility. Thus, the cytotoxicity of κC and AG hydrogels was assessed by an MTS assay, after

culturing L929 cells for 1, 3 and 7 days with the extracts of the materials (Fig. 1). Data obtained from the MTS assay revealed that the metabolic activity of L929 cells after being in contact with the different material extracts varied along the time of the experiment. A decrease in cells metabolic activity (p<0.01), in relation to negative control (100% of metabolic activity), was observed from day 1 to day 3 in the presence of the κCR hydrogel extract, although this decrease was overcome at day 7 (p<0.001). Furthermore, a significantly lower metabolic activity was registered at days 3 and 7 in the presence of κC extracts in comparison to AG extracts (Fig. 2).

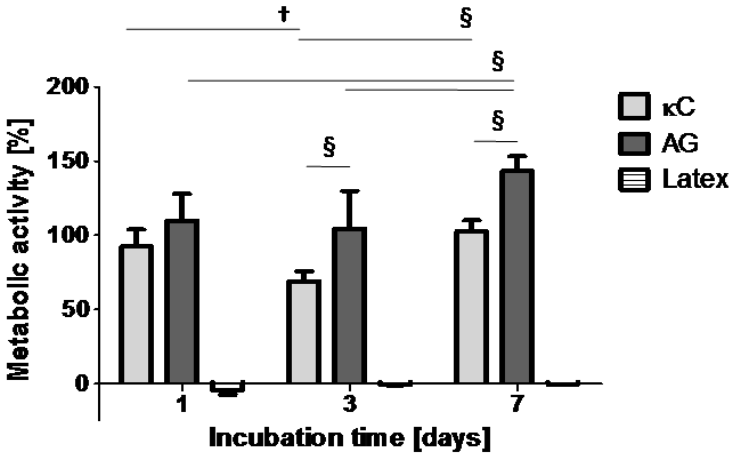

Figure VII- 2. Percentage of metabolic activity in relation to negative control, of L929 cells cultured with leachable from κ-carrageenan (κC) and agarose (AG) hydrogels, and from the latex membranes after 1, 3 and 7 days of culture, data obtained from the MTS assay. The AG hydrogel extract was used as a control material and the extract of latex was used as positive control for cytotoxicity. Statistical analyses were conducted using two-way ANOVA, data was expressed as means ± standard deviation (S.D.); n = 3. Statistically significant differences were register, symbol † for p<0.01 and symbol § for p<0.001.

VII.3.2. Chemiluminescence assay

The effect of κC and AG hydrogels over hPMNs activation was assessed by the detection of the production of reactive oxygen species (ROS) using a chemiluminescence assay. The chemiluminescence results showed that the κC hydrogels (Cells_ κC _Lucigenin), contrarily to AG hydrogels (Cells_AG_Lucigenin, Fig 3c), lead to hPMNs activation and the production of superoxide anion (O_2^-) as detected in the presence of lucigenin. Nonetheless, cells in the presence of the κC hydrogels retained their intrinsic capacity of reaction as demonstrated after the addition of cells stimulant PMA (Cells_ κC _PMA_Lucigenin), condition in which the levels of luminescence were close to the maximum detected after stimulation in the absence of the κC hydrogels (Cells_PMA_Lucigenin) (Fig. 3a). Similar profiles in terms of capacity of reaction after PMA stimulation in the presence (Cells_

κC _PMA_Luminol) and absence (Cells_PMA_Luminol) of κC hydrogels were observed regarding the production of hydroxyl anion ($HO^{\cdot}$), detected in the presence of luminol. However, cells in the presence of the κC hydrogels and without any further stimulation (Cells_ κC _Luminol) do no lead to the production of $HO^{\cdot}$ maintaining along the assay the basal level of activation resulting from the isolation process (Cells_PBS_Luminol) (Fig. 3b) As for the AG hydrogels, although the behavior regarding the production of $HO^{\cdot}$ (Fig. 3d) did not vary from that observed for the κC hydrogels, hPMNs capacity of producing $O_2^{\cdot-}$ after PMA stimulation in the presence of AG hydrogels (Cells_AG_PMA_Lucigenin) was different. Although cells in the presence of the AG hydrogels (Cells_AG_Lucigenin) kept the basal level of activation resulting from the isolation process (Cells_PBS_Lucigenin), after stimulation (Cells_AG_PMA_Lucigenin) the signal corresponding to the $O_2^{\cdot-}$ detection is not only delayed in time, in relation to the potential maximum hPMNs activation profile (Cells_ PMA_Lucigenin), but is also of lower intensity (Fig. 3c) than the one detected in the presence of κC hydrogels (Cells_ κC _PMA_Lucigenin) (Fig. 3a).

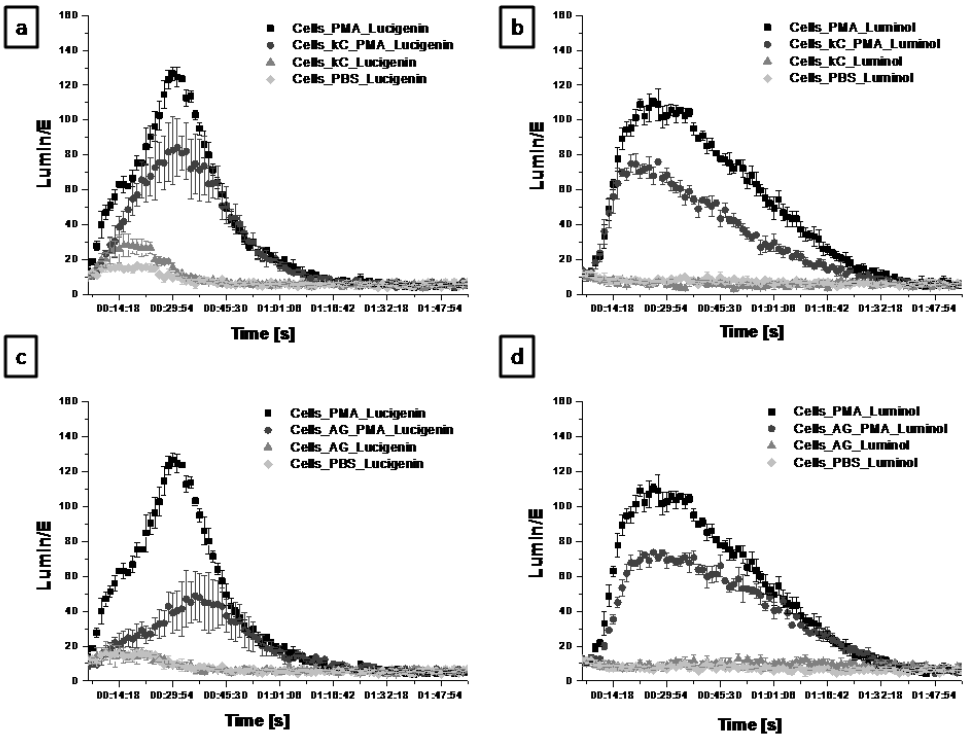

Figure VII- 3. Chemiluminescence measurements of hPMNs activation with and without PMA stimulation, based on the detection of superoxide and hydroxyl anions by oxidation of lucigenin (a, c) and luminol (b, d) respectively, in the presence and absence of κC (a, b) and AG (c, d) hydrogels.

VII.3.3. Explants characterization

The explants, comprising the implanted hydrogels and surrounding tissue, were collected and processed for histological and RT-PCR analysis. At the time of implant retrieval, there were no macroscopic signs of infection, necrosis or swelling in any of the animals and no exudates were formed around the implants. The tissue response to the implanted hydrogels investigated in this study included the characterization of different cell populations responsible for the inflammatory reaction the macrophages – MØ and lymphocytes.

VII.3.3.1. *HISTOLOGY – H&E*

The local and systemic host responses to the implanted hydrogels were analyzed after histological processing of the explants comprising the implanted κC and AG hydrogels and respective surrounding tissue, and of the axillary and inguinal lymph nodes, respectively. Paraffin wax embedded sections stained with H&E confirmed absence of edema and necrosis for all conditions (Fig. 4).

After 7 days of subcutaneous implantation, tissue sections corresponding to the place of implantation of κC hydrogels presented moderate signs of inflammation essentially characterized by the presence of PMNs (Fig. 4). The stained histological sections exposed that, after 7 days of implantation, the AG hydrogels maintained its integrity contrarily to the κC hydrogels, which were completely disintegrated (Fig. 4). At day 7, in the explants sections corresponding to the negative control group, defined by the rats with empty pockets (EP) the presence of vascularized adipose tissue was noticed. The experimental groups showed the presence of an inflammatory infiltrate characterized by the presence of PMNs, macrophages and also blood vessels representative of an acute persistent reaction.

At day 15 days post-implantation the inflammatory infiltrate is mainly characterized by MØ for all the groups (Figure 4), continuing the phagocytic work started by PMNs at earlier stages of the inflammatory process. The extension of the observed inflammatory infiltrate was slightly higher in κC than in AG hydrogels, demonstrating that the former elicited higher recruitment of inflammatory cells in comparison to AG (Fig. 4). At this stage, it is clear the degradation of AG hydrogel by the cells, while the presence of κC hydrogels material is not detected for the same time analyzed (Fig. 4). Foreign body giant cells, one of the typical features of chronic inflammation, were not observed.

In terms of systemic reaction, the lymph nodes at 7 days depicted vascular channels, a mixed inflammatory infiltrate composed of fibroblasts, neutrophils and lymphocytes, and fibrous stroma of the node, independently of the tested group. Higher presence of the inflammatory infiltrate cells were detected at day 15 especially for κC and LPS group. Nonetheless, after 2 weeks, the analysis of the

explanted lymph nodes after H&E staining revealed the absence of germinal centers in their cortex (Fig. 5).

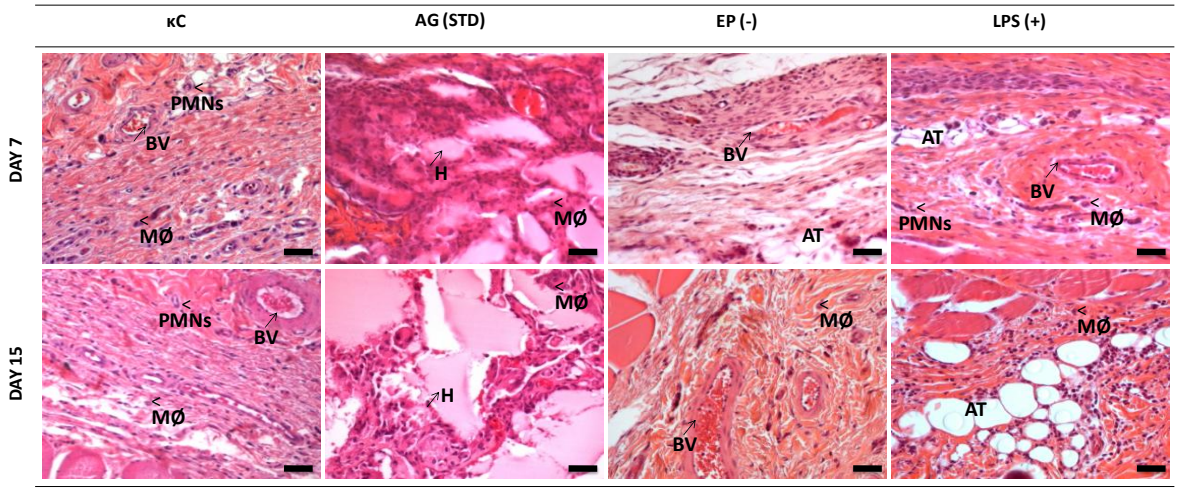

Figure VII- 4. Micrographs of H&E staining of explants sections obtained after 7 and 15 days of subcutaneous implantation of κC, AG hydrogels. The negative control group corresponds to the empty pockets (EP (-)) while the positive control (LPS (+)) corresponds to the group injected with LPS 24 hours before the defined time point. The abbreviations corresponds to BV - blood vessels; PMNs – polymorphonuclear neutrophils; MØ - macrophages; AT - adipose tissue; H - implanted hydrogel.

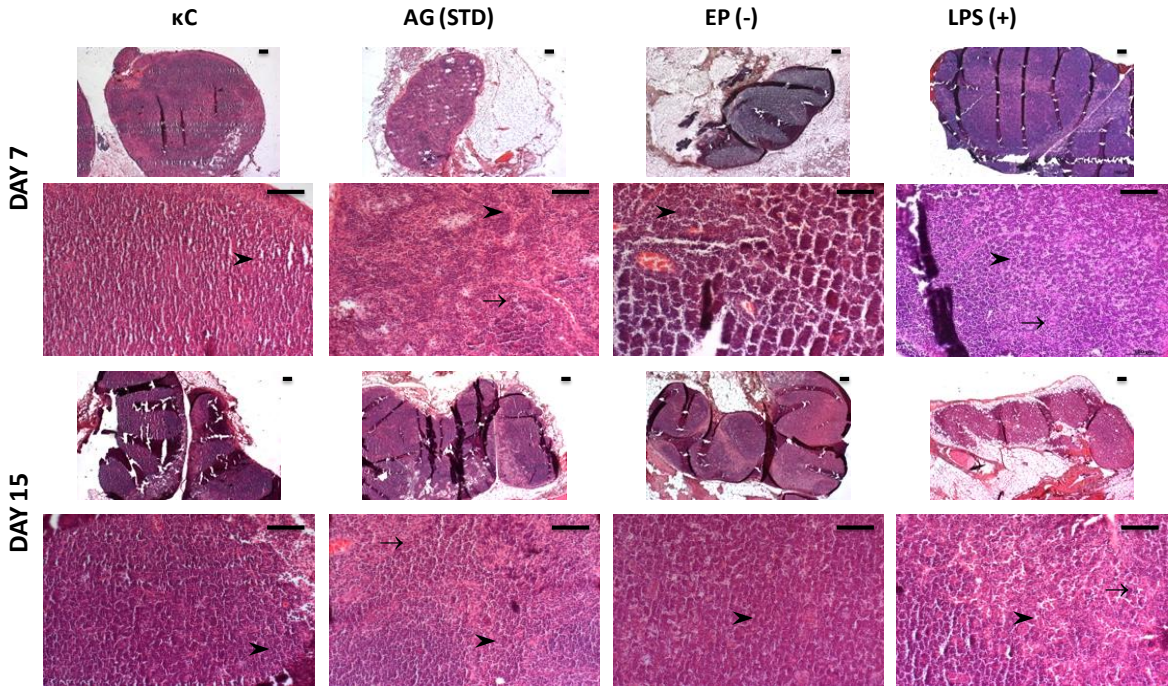

Figure VII- 5. Micrographs of the lymph nodes, stained with H&E, explanted from the κC, AG, EP, LPS groups after 7 and 15 days of subcutaneous implantation; the scale bar corresponds to 100 μm. Medullar region of the lymph nodes are represented in higher magnification images. Arrowhead indicates lymphocytes and long thin arrow designate neutrophils.

VII.3.3.2. *Macrophages recruitment and lymphocytes activation*

The CD163 immunodetection confirmed the presence of mainly recruited macrophages, while CD25 immunostaining showed few positive activated lymphocytes, after one week of subcutaneous implantation (Fig. 6). The densities of the inflammatory cells seem to increase with time; while M2 macrophages seem to be recruited in significant numbers in both experimental (κC) and control material (AG) groups, activated lymphocytes were mostly identified after implantation of κC hydrogels.

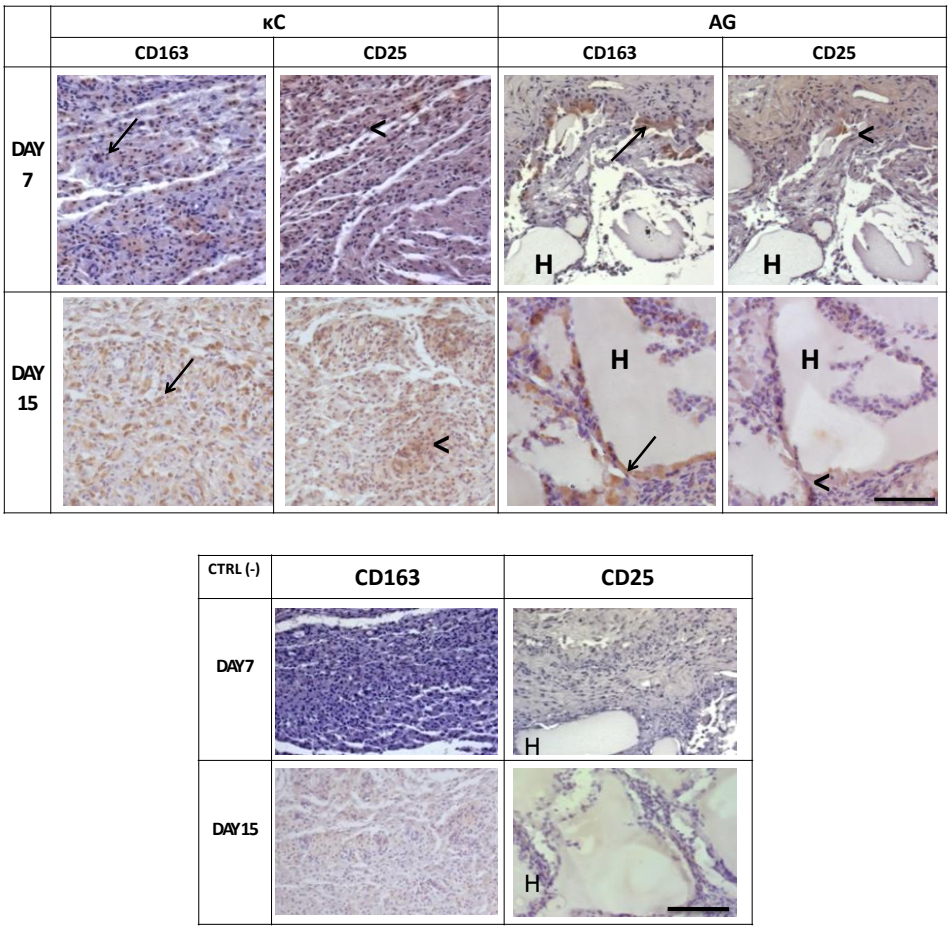

Figure VII- 6. Micrographs of 7 and 15 days post-implantation explants after immunohistochemical labelling for recruited macrophages (CD163) and activated lymphocytes (CD25). Scale bar corresponds to 100µm. Arrows indicate positive cells for the CD163, arrow heads represent positive staining for CD25 and H is the abbreviation for implanted hydrogel.

VII.3.3.3. *Analysis of pro-and anti-inflammatory genes expression*

The expression profile of pro- and anti-inflammatory within the resolution of an inflammatory process is expected to result in a pro-wound healing pattern of cytokines. The expression of IL-1 alpha and IFN-gamma, along with the absence of IL-4 was observed at day 7 of subcutaneous implantation of both

hydrogels (Fig.7). At day 15 of implantation it while IL-1 alpha expression was still detected, both the absence of expression of IL-4 and IFN-gamma were observed.

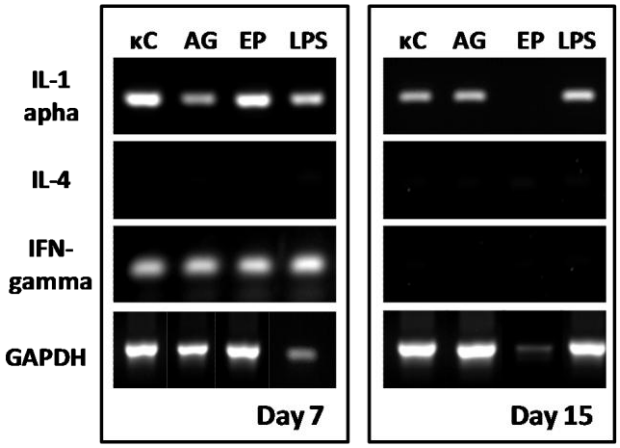

Figure VII- 7. *IL-1 alpha*, *IL-4*, *IFN- gamma*, *GAPDH* expression analysed by RT-PCR and visualized on a 1.7 % agarose gel stained with ethidium bromide. (Left to right) Lanes 1–4: 7 days after subcutaneous implantation; lanes 5–8: 15 days after implantation.

VII.4. Discussion

In has been reported that carrageenan sulfated polysaccharides have diverse biological activities derived from their chemical composition (varying amounts of ιota, κappa or lambda carrageenan precursors), sulfate content, molecular weight and extraction procedures [30, 31]. Moreover, the structural similarity of carrageenan, based on sulfated galactose or modified galactose residues, with components of the extracellular matrix, namely glycosaminoglycans (GAGs), might lead to specific biological reactions [32]. Carrageenan biocompatibility, including their immunomodulatory role has been discussed in several studies [33-35]. Most of these studies were centered in the use of *lambda*-carrageenan solution and typically they lack data regarding dose, type of carrageenan or seaweed source, making it difficult to establish consensus among the reported results [36-39]. In this context it was essential to evaluate the newly developed hydrogels in terms of biocompatibility starting with *in vitro* testing followed by *in vivo* assessment, thus allowing understanding as much as possible carrageenan cytotoxicity and tissue compatibility.

The metabolic activity of L929 cells exposed to hydrogel κC extract indicated that this natural origin polysaccharide does not induce a harmful effect on cells demonstrating the extremely low cytotoxic levels of the κC hydrogels. Similar results were observed for the AG hydrogel extract although a significantly higher metabolic activity, in comparison to κC hydrogels, was registered at day 7. A possible explanation for these results may be the difference in the gelation and the degradation

mechanism of the two polysaccharides. κC hydrogels are thermosensitive and ionic hydrogels while the gelation mechanism of AG is fully temperature dependent. The degradation of κC hydrogels when subjected to ionic environment such as culture medium can alter the stability of the gel, following its dissolution. Therefore, since κC degradation is faster, it is possible that a higher amount of degradation products are released during the extraction procedure, thus affecting the cell metabolic activity. Moreover, the cell behavior may also be influenced by potassium ions, the gelation agent, released from the hydrogels.

Human polymorphonuclear neutrophils are crucial in the early development of an inflammatory response and dictate the progression of the host reaction [29, 40]. Typically, hPMNs experience a respiratory burst upon activation, releasing ROS that aim to eliminate the foreign inflammatory agent. ROS have been implicated in several key cellular processes, including oxidative stress damage [41] tumor promotion [42] and most recently, cell growth and DNA synthesis [43], but at high concentration can damage the cell structures [44]. Thus, assessing the release of ROS *in vitro* by placing hPMNs in contact with κC hydrogels is a way to predict, with the limitations of any other *in vitro* system, the initial material-host interactions that occur upon implantation. When hPMNs were in contact with κC hydrogels, a small amount of O_2^- anions were detected but no signal corresponding to luminol oxidation by OH^- anions was observed. Moreover, cells were able to retain their intrinsic capacity to produce both ROS as demonstrated by the increased signal observed under the same conditions but in the presence of PMA stimulation. Nonetheless, the intensity of the detected signal was lower in the presence of the κC hydrogels, and slightly shifted in time in the case of the O_2^- detection. While the diminished intensity might be consequence of the interaction/scavenging of the ROS by the materials, the delayed detection most likely results from the cell-material interactions that interfere with the PMA activation pathway [45]. In fact, an even more pronounced signal was observed in the presence of AG hydrogels and upon hPMNs PMA activation and in particular in the detection of the O_2^- anions. This suggests a different interaction between hPMNs and the studied hydrogels, naturally arising from their distinct nature, and consequently a potentially different *in vivo* response. It has been shown, that hPMNs attachment to surfaces alters not only their shape but modulates their activity with regard to ROS production [46, 47]. It is therefore expected that direct hPMNs-hydrogel interactions contribute to the initial respiratory burst which, considering the obtained results, is not foreseen as factor that might contribute to an intense inflammatory reaction.

This was in fact confirmed after subcutaneous implantation of the studied hydrogels as no physiologic signs of severe inflammation were observed. The typical reaction characterized by the presence of PMNs and macrophages was observed for both κC and AG hydrogels. The materials induced an influx

of neutrophils at early time points aiming at phagocytosis the foreign body which then progressed into an inflammatory infiltrate mainly composed by mononuclear cells, i.e. macrophages, lymphocytes and some fibroblasts. Administration of carrageenan has been reported to interfere with the phagocytic activity [48] and to be cytotoxic for macrophages in a concentration-dependent manner. The degradability of κC hydrogels is mostly based on hydrolysis of either the cross-linker or the polymer backbone [49] as this natural polymer requires the presence of ions for the formation of a stable hydrogel. These physically cross linked hydrogels tend to lose their stability after implantation due to the exchange of monovalent with divalent ions that are present in higher concentrations in physiological environments, as discussed for other systems [50]. Moreover, it is possible to convey that κC was degraded by matrix metalloproteinases secreted by the cells from the implantation site surroundings [51]. The absence of necrosis at the κC hydrogels implantation sites seems to indicate that macrophages were able to engulf the hydrogels, even showing higher degradation rate than AG hydrogels, within a context of a moderate inflammatory process. Moreover, the analysis of the lymph nodes after κC implantation did not indicate an activation state or an induced systemic host reaction. Apparently, no molecules of the materials were transported throughout circulation to induce a remote response.

Macrophage phenotype has been characterized as pro-inflammatory (M1) or immunomodulatory and tissue remodeling (M2). These, specifically express the CD163 surface marker and are commonly associated to the release of anti-inflammatory cytokines [52]. Macrophages expressing CD163 have been identified in significant numbers at the implantation sites of both hydrogels though a higher number seems to be present in the κC hydrogel group in comparison to AG hydrogel, especially at day 15 post implantation. This can be explained by the differentiated progression of the inflammatory process that is likely to be more advanced, thus expected to be resolved faster, in the κC hydrogel group due to their earlier degradation. The presence of AG hydrogels after 15 days of implantation contribute to the persistence of the reaction and to more pro-inflammatory stage characterized by a lower amount of M2 macrophages. IL-1 alpha expression at the different implantation times confirmed the inflammatory character of the reaction to both hydrogels studied. This cytokine is, at early stages of reaction, secreted by macrophages and endothelial cells, then stimulating the activation of B and T lymphocytes [53]. In response to IL-1 alpha secreted by activated macrophages, activated lymphocytes secrete IFN gamma [54]. Interestingly, our results confirm the expression of IFN gamma a day 7 post-implantation but not at day 15, in which activated lymphocytes, CD25 positive cells, were identified. Moreover, the subcutaneous implantation of the hydrogels did not induce IL-4 expression, a T-cell derived inflammatory cytokine that inhibits macrophages response. Thus, at the end of latest

implantation period a typical acute inflammatory process caused by the degradation of the hydrogels occurs. Although the differences observed for the two implanted types of hydrogels were tenuous, the resolution of the inflammatory process is expected to be faster for the κC hydrogels group.

VII.5. CONCLUSIONS

The results presented in this study show that κC extracted proved to be cytocompatible, and did not significantly promote the stimulation and the respiratory burst of hPMNs. The inflammatory reaction observed after subcutaneous implantation of the materials, represented a typical acute response upon injury. The dissolution of the κC hydrogel components did not lead to the formation of toxic products and the biomaterial could be readily resorbed or excreted. Based on these observations it is possible to establish that κC hydrogels can be further studied for medicine regenerative applications, particularly for systems aiming at fast cell/bioactive molecules delivery.

Acknowledgments

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Chapter VIII. PRELIMINARY EVALUATION OF THE IN VIVO BEHAVIOR OF K-CARRAGEENAN HYDROGELS LADEN WITH HUMAN ADIPOSE DERIVED STEM CELLS

Preliminary evaluation of the in vivo behavior of κ-carrageenan hydrogels laden with human adipose derived stem cells

ABSTRACT

Recent studies have shown that human adipose derived stem cells (hASCs) loaded in κ-carrageenan hydrogels promote *in vitro* formation of cartilage-like tissue that exhibits increased mechanical properties with time in culture. These results show that κ-carrageenan holds adequate properties to enable the *in vitro* functionality of encapsulated hASCs, positioning them as valid alternatives to hydrogels currently used in cartilage regenerative strategies. Nevertheless, *in vivo* studies revealed a fast degradation of the κ-carrageenan hydrogels (without cells), upon their subcutaneous implantation in a rat model.

Therefore, the present work was designed to assess the behavior of the κ-carrageenan hydrogels either freshly laden with hASCs or pre-cultured in chondrogenic medium, as compared to the hydrogels alone. The hydrogels were produced by an ionotropic gelation method and human adipose stem cells were encapsulated in a 1.5 % (w/v) κ-carrageenan solution at a cell density of $5x10^6$ cells/mL^{-1}. hASCs laden hydrogels were subcutaneously implanted in rats, either right after encapsulation or after pre-culture in chondrogenic medium for 1 week. Explants were analyzed by histological techniques, revealing normal cell morphology, and suggesting that the presence of cells improves the stability, preventing the faster degradation of the hydrogels, in agreement with previous *in vitro* outcomes and supporting the claim that hASCs laden κ-carrageenan hydrogels hold a great potential in the field of cartilage regeneration.

Keywords: adipose derived stem cells; cartilage; chondrogenic differentiation; hydrogels; κ-carrageenan.

VIII.1. INTRODUCTION

Cartilage is a connective tissue composed of extracellular matrix (ECM) on which the chondrocytes are embedded, characterized for being avascularised and for presenting a low metabolic rate [1-3]. The limited regeneration capacity of this tissue can create disability states associated to pain and decreasing of the quality of the life [3]. Several strategies have been pursued in order to replace the degenerated cartilage, but none of them was considered to be fully satisfactory [4-6]. Tissue engineering is considered the most promising alternative approach to develop cartilage regeneration therapies based on the use of biomaterials and cells, sometimes combined with bioactive agents [7, 8]. Several materials of natural origin have been studied and proposed to support cells development and functional cartilage tissue formation [9-18]. The use of hydrogels has gathered a strong importance in this research field, mainly because they can be injected into the tissue defect, adapting to the precise geometry and allowing for an higher safety of the implantation procedure [19]. Additionally, hydrogels obtained from natural origin polymer have also shown to enable the adequate functionality of encapsulated cells and therefore providing suitable matrices for their *in vivo* delivery [20]. Recently, carrageenan polysaccharide was proposed by the 3B's research group for application in the cartilage tissue-engineering field [21, 22] owing to its advantageous properties in comparison to other hydrogels, namely the ionic and temperature dependent gelling mechanism and chemical composition [23, 24]. Cells constitute one of the fundamental components of any cartilage tissue engineering approach. Although primary autologous chondrocytes have been widely used for the regeneration of cartilage, adipose tissue derived stem cells (ASCs) have promising future application in this field, mainly due to their wide availability in the human body, easiness of the harvesting procedures, the high number of stem cells and for their great chondrogenic differentiation potential [25-35].

In a previous study [24], it was shown that, due to the ionic nature of κ-carrageenan, the amount of ions present in the medium significantly affects the hydrogels swelling properties and ultimately the degradation profile of the hydrogel. Moreover, the *in vitro* culturing of κ-carrageenan hydrogels laden with stem cells in chondrogenic medium increased the mechanical properties of the constructs along with time in culture time, suggesting that the increased extracellular matrix production is responsible for enhanced mechanical properties and progressive maturation of the tissue formed *in vitro*. However, when κ-carrageenan hydrogels without cells were subcutaneously implanted, revealed their low stability *in vivo*. Therefore, the aim of this study was to evaluate if the encapsulation of cells could improve the *in vivo* behavior of the developed κ-carrageenan hydrogels. For this purpose, the cell laden hydrogels,

with or without pre-culture in chondrogenic medium, as well as hydrogel with no cells, were implanted in a rat subcutaneous model for 7 days and the explants evaluated by histological techniques.

VIII.2. Materials and Methods

VIII.2.1. κ-Carrageenan hydrogel preparation

An aqueous solution was prepared by dissolving the κ-carrageenan powder (22048, Sigma Fluka) in distilled water, and heating at 60 °C while stirring constant until complete dispersion. The κ-carrageenan solution was autoclaved during 30 minutes at 120 °C before use, with a final concentration of 1.5 % (w/v) and 5 % (w/v) of potassium chloride (KCl, P5405, Sigma). The hydrogels discs were formed using 48 well plates and KCl for 10 minutes, in order to stabilize the 3-dimensional structure. Afterwards, the gels with dimensions of 11.5 ±0.01 mm x 1.8 ±0.24 mm height were washed with phosphate buffered saline (PBS, D5652, Sigma) for further use.

VIII.2.2. Human adipose derived stem cells isolation and expansion

Human liposuction aspirate samples were obtained upon patient informed consent from donors undergoing lipoaspiration procedures under a protocol established with the Department of Plastic Surgery of the Hospital da Prelada, Porto, and approved by the local Ethical Committee. The human adipose derived stem cells (hASCs) were enzymatically isolated as previously described [36]. Briefly, the adipose tissue samples were digested with 0.2 % collagenase type II (C6885, Sigma) in PBS for 45 min at 37 °C under gentle stirring. The digested tissue was filtered, centrifuged at 1200 rpm for 10 min at 20 °C and washed 5 min with lysis buffer to remove erythrocytes. The supernatant was removed and cells were resuspended in alpha Minimum Essential Medium (α-MEM, 12000-063 Gibco, Invitrogen) with 10 % FBS (10270-106 Gibco, Invitrogen; heat inactivated), 1 % Antibiotic-Antimycotic (15240-062, Invitrogen) and sodium bicarbonate (S5761-NaHCO3, Sigma). Human ASCs were plated at a density of 3.5 x 10^3 cells/cm^2 and incubated at 37 °C in a humidified atmosphere of 5 % CO2.

VIII.2.3. Encapsulation of hASCs in κ-carrageenan hydrogels

Cells were resuspended in κ-carrageenan 1.5 % (w/v) solution for complete homogenization with a final concentration of 5x10^6 cells·mL^{-1}. Hydrogel discs containing human ASCs were prepared as previously

described. After preparation the discs with encapsulated cells were cultured in chondrogenic differentiation medium for 7 days. Hydrogels disc with hASCs pre-cultured or κ-carrageenan laden with cells freshly prepared, were subcutaneously implanted for 7 days. Additional controls consisted of κ-carrageenan hydrogel samples without cells and empty defects were created. The chondrogenic differentiation medium was composed of Dulbecco's Modified Eagle's Medium- low glucose (DMEM, D5523, Sigma), supplemented with 10 % FBS (10270-106 Gibco, Invitrogen), 1% Antibiotic-Antimycotic (15240-062, Gibco, Invitrogen), ITS+1 Liquid Media Supplement (I2521- insulin-transferrin-selenium - liquid media supplement, Sigma), 17 mM L-ascorbic acid (A4544, Sigma), 0.1 M sodium pyruvate (P4562, Sigma), 35 mM L-proline (P5607, Sigma), 1 mM dexamethasone (D4902, Sigma) and 10 ng/ml of human TGF-β1 (Transforming Growth Factor-β1, 14-8348, eBioscience).

VIII.2.4. Subcutaneous implantation of cell loaded κ-carrageenan

Surgeries were made on 9-week-old male Wistar rats under standard sterile conditions. Briefly, the rats were anesthetized with an intraperitoneal injection of a combination of medethomidine (30-100 μg/kg) plus ketamine (50-100mg/kg). After shaving the hair and disinfection with povidone-iodine (Betadine), a longitudinal incision was made on four distinct regions of the back of the rats. Ionic κ-carrageenan hydrogels with and without cells were subcutaneously implanted. Empty defects were used as controls. Then, the wounds were closed with a nonabsorbable polypropylene suture 4-0 (Figure 1). All the animal experiments were performed accordingly with the National Ethical Committee for Laboratory Animals and conducted in accordance with Portuguese legislation (Portaria n°1005/92) and international standards on animal welfare as defined by the European Communities Council Directive (86/609/EEC). Each rat received the implantation of 4 constructs randomly placed on their back, as represented in figure 1. Animals were placed on a heating pad under a warming light and observed until they recover consciousness. The rats were assessed for 30 minutes following their return to consciousness to assess evidence of distress and placed in cages (2/3 per cage). Animals received analgesia preoperatively (lidocaine) and during the post-operative period (carpofen) as needed based on evidence of discomfort. This was evaluated by animal behavior, feeding, and vocalization. Sutures were removed after 7 days postoperatively and animals were euthanized by asphyxiation with CO_2. The explanted tissues were fixed in 4% formalin, embedded in paraffin, sectioned and processed for histological analysis.

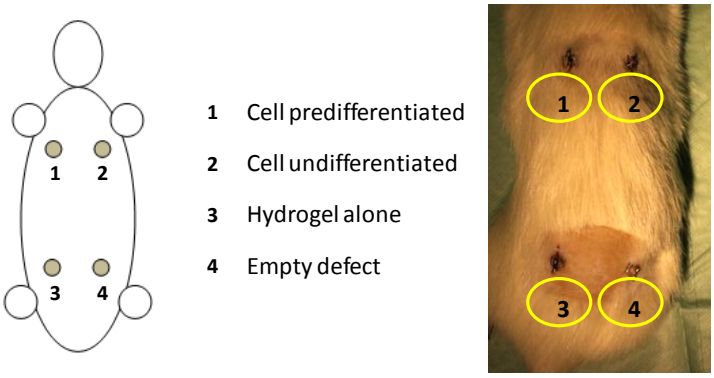

Figure VIII- 1. Schematics of the implantation of four individual subcutaneous pockets, created by blunt dissection, with the different studied groups.

VIII.2.5. Histological analysis

Samples were collected at the end of the pre- fixed experimental endpoint (7 days), dehydrated and embedded in paraffin. Sections were cut at 4 μm and placed on microscopy slides. Hematoxylin-eosin (H&E) and Masson Goldner Trichrome (MGT) staining were performed. For H&E staining, after hydration the sample sections were colored with Papanicolaou Harris hematoxylin (05-12011/L, Bio-optica) for 3 minutes, washed in running tap water and afterwards a blue stain enhancement was performed by an immersion in 0.5 % ammonia (05002, Sigma) for 5–10 seconds. The sections were washed in running tap water and stained in Eosin-Y (05-M10003, Bio-optica) for 30 seconds. For MGT different stains were used: Weigert's iron hematoxylin for nuclei, picric acid for erythrocytes and light green for collagen. After typical hydration steps, Weigert's iron hematoxylin reagent was added to the sections and left to act for 15 minutes. Without washing, the slides were drain and picric acid alcoholic solution was added to the section for 4 minutes. After quick wash in distilled water the other reagents were put on the section to act for a total of 15 minutes. Finally all slides were dehydrated through series of alcohol immersions from 30 % until 100 % alcohol. The final step for all the staining performed was the immersion in the clearing agent Histoclear® (National Diagnostics) or xylene substitute for 1-2 minutes and mounted using Microscopy Entellan® (Merck &Co., Inc.) for later observation. Stained sections were observed under a light microscope (Reflected/Transmitted light Microscope, Zeiss).

VIII.3. Results and Discussion

κ-Carrageenan hydrogels have been previously proposed for applications in cartilage regeneration since they enable to encapsulate/deliver cells under mild conditions [21, 24]. In these studies, cells have been encapsulated in κ-carrageenan hydrogels and cultured *in vitro* for extensive periods of time. The cells were viable, proliferated and formed a hyaline-like ECM composed of collagen type II. Figure 2 shows human adipose derived stem cells efficiently encapsulated in the κ-carrageenan hydrogels and homogeneously distributed, exhibiting a round-shaped morphology typical of native human chondrocytes. The rounded shape was more obvious when stem cells were cultured in the presence chondrogenic differentiation medium assuming a chondrogenic phenotype. Although carrageenan is sensitive to mediums rich in electrolytes, such as physiological environment, destabilizing the hydrogel, previous in vitro studies have shown that the stiffness and the stability of the structure was increased with cell loading, [24].

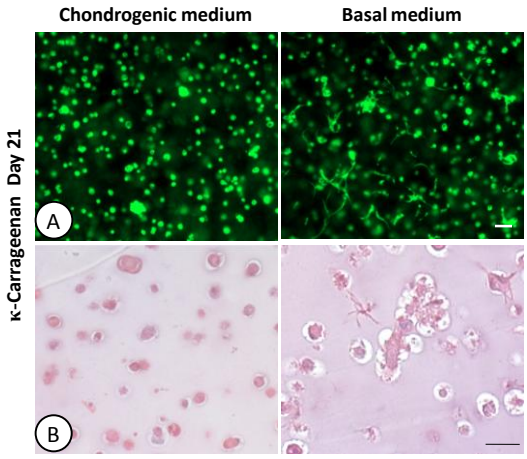

Figure VIII- 2. A - Calcein AM staining of human adipose stem cells (hASCs) encapsulated in κ-carrageenan hydrogels cultured in chondrogenic and basal medium. Fluorescent images showing the viability and distribution of encapsulated hASCs after 21 days of culture, magnification 5x, with 100 μm scale bar. B - Haematoxylin&Eosin staining of hASCs encapsulated in κ-carrageenan hydrogels, exhibiting vacuoles morphology and cell clusters formation, scale bars have 100 μm and images are taken at 20x magnification.

Nevertheless, upon subcutaneous implantation of the hydrogels without cells, the evaluation revealed low stability/fast degradation of the hydrogels with time. Thus, in this study it was evaluated the stability of the hydrogels once cells (hASCs) were loaded and, in some cases, pre cultured in chondrogenic media. Cell-laden hydrogels have a higher stability due to the increase in ECM deposition

within the hydrogels, as observed *in vitro*. As such, κ-carrageenan hydrogels discs with cells were subcutaneously implanted in wistar rats and histological analysis was conducted upon harvesting the explants, 1 week after the surgeries. Upon explantation, no evident macroscopic changes of the hydrogel samples and surrounding tissues were observed and the samples maintained its structural integrity.

Figure 3 shows different histological sections of explants corresponding to the cells laden κ-carrageenan hydrogels, obtained upon 7 days of subcutaneous implantation. Hematoxylin-eosin staining performed provided relevant information on cell morphology and distribution within the hydrogel matrix. The histological analysis of the explanted samples showed good integration within the surrounding host tissue. The observations also indicate that explants correspondent to hydrogels laden with cells exhibited higher cell density when compared to hydrogels alone. The presence of the cells in the hydrogels seems to have created consistent adhesion interface with the surrounding tissue, as can be observed in figure 3. κ-Carrageenan alone and the empty pocket (EP) presented similar morphologies, exhibiting good integration/ingrowth of surrounding tissues, although this feature was detected more frequently in κ-carrageenan hydrogels.

It is clear t that the presence of cells increases the stability of hydrogels, providing further evidence that the subsequent deposition of extracellular matrix components within the hydrogel, facilitated by the gradual degradation of the gels, by it turn increases their stability. It has been proved that the biomechanical properties of articular cartilage greatly depends on composition, density of its ECM and interstitial fluid flow (water and solutes) [37]. So, cells encapsulation and extracellular matrix deposition may result in progressive increase of the mechanical properties of the 3D structures, as shown before for the cell laden hydrogels cultured in vitro [38]. The cells incorporated in a matrix are expected to confer an improved advantage in new tissue formation and integration due to the role they can develop in tissue regeneration, both directly or indirectly, through the production/release of soluble factors that participate in these processes.

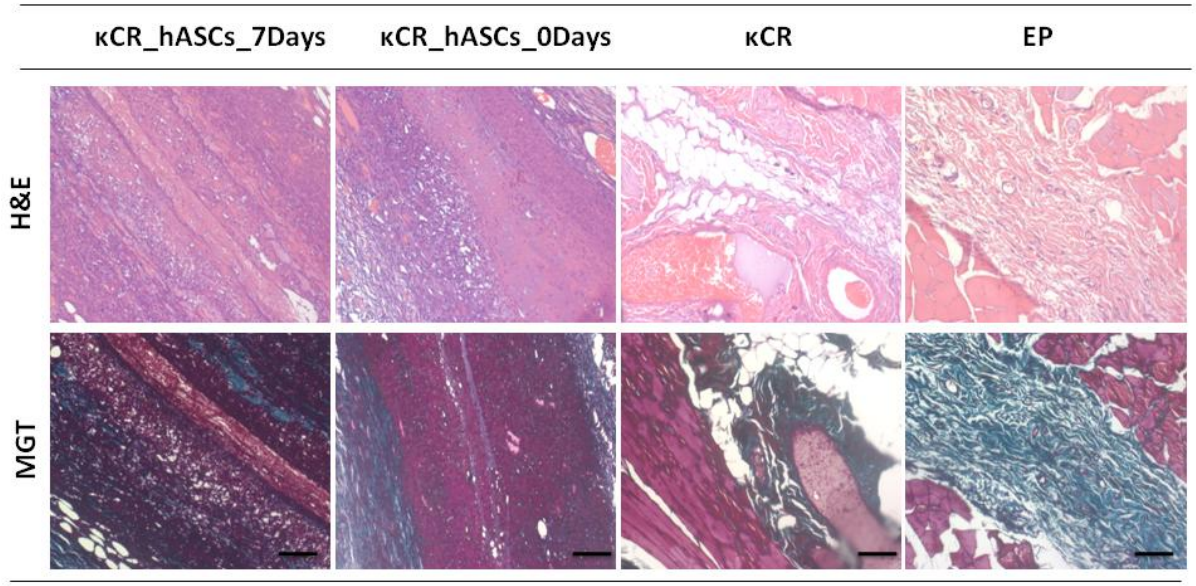

Figure VIII- 3. Micrographs of the sections of the explanted κ-carrageenan hydrogels loaded with predifferentiated for 7 days and undifferentiated cells stained with H&E and MGT. The images shown correspond to samples collected after 7 days of culture.

VIII.4. Conclusions

This work presents κ-carrageenan as a potential hydrogel for cell delivery with application in the regeneration of cartilage. The results obtained using κ-carrageenan hydrogels as cell loading matrix, prepared via ionic cross linking reaction, demonstrate that this system could be an alternative cell delivery hydrogel systems. The results clearly demonstrate the positive influence of the systems laden with cells, showing that human adipose stem cells encapsulated in κ-carrageenan hydrogels pre-differentiate into the chondrogenic lineage, forming ECM like matrix that is responsible for higher stability of the final constructs.

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SECTION 4. CONCLUSION AND FINAL REMARKS

Chapter IX. CONCLUSIONS AND PERSPECTIVES

IX.1. GENERAL CONCLUSION

Current clinical repair methods cannot restore original properties nor promote the full regeneration of native cartilage tissue and consequently a great deal of efforts regarding the development of more efficient solutions, based on tissue engineering and regenerative medicine concepts, was the motivation background in this thesis.

In native cartilage tissue, cells are embedded within the extracellular matrix (ECM) therefore, using hydrogels to encapsulate cells seems as an obvious choice as they provide an hydrated 3D environment that can be designed to mimic the composition/properties of the native ECM. Several natural origin hydrogels have been widely investigated for cartilage tissue engineering (TE) applications yet, an optimal material is still to be found. Carrageenan hydrogels offers great potential for such purpose due to its specific properties, but so far, almost unexplored in the biomedical field. Some of the advantages that such hydrogels may bring over current systems include the thermoreversible and ionic hydrogel characteristics that enable mild condition for cell encapsulation purposes and versatility of processing, obtaining various shapes/formats. The resemblance of their chemical structure to the glycosaminoglycans that compose the ECM of tissue and the presence of functional groups allow them to be easily chemically modified, rendering improved functions. Therefore, the main objective of this work was to develop and optimize carrageenan based hydrogels systems with adequate properties for the encapsulation/delivery of cells targeting cartilage repair strategies based on TE concepts. Thus, the work described also focused on the assessment of the *in vitro* chondrogenic potential of such systems laden with different cell types, as well as on their *in vitro* and *in vivo* biocompatibility evaluation.

The work developed under the scope of this thesis allowed to obtain several conclusions that are be detailed bellow. The majority of the work herein described was centered on κ-carrageenan based systems; however, the first chapter concerning experimental work focused on the development and characterization of new hydrogel systems based on alginate and two different types of carrageenan, processing these blends into different formats and optimizing their properties to sustain the viability of encapsulated cells. Several different aspects were considered, such as, the use of different types of carrageenan (ιota and κappa), different concentrations of the polymers and various concentrations of the salts used for the ionic gelation. Spherical beads have been obtained by an extrusion technique and the fibers were prepared using a coagulation bath. Alginate was found to be inappropriate for applications that required defined shape due to their fast gelation and inadequate mechanical strength. Carrageenan showed to be able to withstand higher loads under compression and a gelation time

suitable for cell encapsulation. Assessment of the viability and proliferation of ATCD5 chondrocytes encapsulated onto the developed beads and fibers after different periods of culturing for 1, 7, 14 and 21 days, revealed enhanced outcomes for κ-carrageenan hydrogels in comparison to ι-carrageenan, probably related to the higher content of ester sulphate groups along the polymer chain. Overall, the stability and the biological behavior of the beads and fibers were mostly influenced by the concentration and ratio of the polymer mixture.

In the study carried out in chapter IV, it was determined the physical characteristics, such as swelling and mechanical properties of the carrageenan hydrogels. The hydrogels exhibited a pH-dependent swelling kinetics and it was found that the amount of ions present in the medium also affects significantly the hydrogels swelling properties. The mechanical properties of these hydrogels, as shown by the storage moduli (0.2 hydrogel alone and 0.4 MPa when cells were laden) were fairly positive when compared to other natural origin hydrogels considering the envisioned applications. In addition, the *in vitro* culturing of human adipose derived stem cells (hASCs) incorporated within the hydrogels led to the formation of a cartilaginous matrix rich in proteoglycans and collagen type II, showing evidences of commitment of the encapsulated cells towards the chondrogenic pathway. The extracellular matrix production seems to be responsible for the increase in the dynamic mechanical properties of the cell laden hydrogels, providing further evidence of their suitability for cartilage tissue engineering.

Along with the initial evaluation of the hydrogel, the assessment of the *in vitro* chondrogenic potential of κ-carrageenan hydrogels laden with different cell types namely, a chondrocytic cell line, primary chondrocytes cells and hASCs, often proposed for cartilage regeneration strategies, was performed in chapter V. Several studies demonstrated that different cell types have variable responses to identical embedding materials due to the different cellular metabolism. Cell lines for example, divided more quickly, primary cells and stem cells are more sensitive with differences in doubling times, or number. Moreover limitations associated with the harvesting (availability, additional surgical intervention, donor site morbidity) isolation and *in vitro* proliferation procedures (chondrocytes easily dedifferentiate) must be carefully considered when selecting an optimal cell type for use in a tissue engineering approach. Overall, the biological evaluation of κ-carrageenan hydrogel revealed that this polymer enables long term viability and proliferation of different cells. During 3 weeks of culture, all cells types encapsulated within the hydrogel developed a cartilage-like extracellular matrix rich in proteoglycans and type II collagen, indicating a stable chondrocyte phenotype. However, encapsulated hASCs exhibit the highest proliferation rates and highest levels of chondrogenic markers expression, as compared to the other cell types studied. These findings encourage both the use of κ-carrageenan hydrogels as cell

matrices/delivery carriers and the use of hASCs for applications in the regeneration of articular cartilage defects.

One of the main prospects of cartilage tissue engineering is the possibility of developing custom-made autologous regenerative medicine solutions on an individual patient basis that can offer also "off the shelf" availability for application upon an immediate clinical need. Therefore, the development of efficient preservation and storage procedures will provide products available as needed, which could be adapted to an autologous immediate solution. Thus, the study in chapter VI aimed to examine the effects of cryopreservation on the chondrogenic differentiation of hASCs encapsulated in κ-carrageenan hydrogels. The developed systems maintained their mechanically stability and cellular viability upon exposure to lower temperatures and the assessment of chondrogenic features indicated a fair chondrocyte phenotype after cryopreservation. In summary, hASCs-hydrogel constructs have high long term storage potential and a ready-to-use bioengineered tissue substitutes for cartilage regeneration strategies. Thus, cell encapsulation systems of natural based hydrogels may be an interesting approach for the long term preservation of cartilage tissue engineered products.

The development of new biomaterials requires extensive biological testing to demonstrate the safety and the functionality of the material and its degradation components and/or incorporated cells. Thus, chapter VII was devoted to investigate the *in vitro* and *in vivo* biocompatibility of the developed system, including *in vitro* experiments with human polymorphonuclear cells and the *in vivo* evaluation of the host reaction to the subcutaneous implantation of κ-carrageenan. The cytotoxicity tests performed revealed that the developed hydrogels were nontoxic and did not inhibit cell growth. The *in vitro* biological screening using hPMNs and the *in vivo* subcutaneous implantation designed to assess for the inflammatory reaction, revealed that the materials do not cause severe host response. At implant retrieval there were no macroscopic signs of a considerable inflammatory reaction in any of the animals and no cellular exudates was formed around the implants.

The final stage of the work described in this thesis involved the *in vivo* assessment of loaded stem cells pre-differentiated or stem cells non-differentiated in κ-carrageenan hydrogels addressing fundamental questions concerning the *in vivo* degradation of the hydrogel and possible *in vivo* differentiation of encapsulated cells. The findings suggest that with addition of cells, the stability and the stiffness of κ-carrageenan hydrogels was improved, as previously found from the *in vitro* studies.

In summary, the overall results showed to be very promising for all types of cells encapsulated in carrageenan based hydrogels, positioning them as valid alternatives to commonly used standard hydrogels. Moreover, knowing that κ-carrageenan forms a hydrogel with potassium ions and the temperature-induced gelation enables its application as a cell-carrier or as an *in vivo* injectable system.

IX.2. Outlook and Perspectives

Herein it was reported that ionic hydrogels based on carrageenan provide easy and inexpensive methods for producing cell friendly matrices, which enable stem cells differentiation into chondrocytes-like cells, producing cartilage ECM components. Even though the outcomes of the described studies clearly demonstrate the potential for successful application of carrageenan hydrogels loaded with human adipose derived stem cells, additional research directions are considered fundamental to consolidate the merits of such approach for cartilage regeneration.

For example, further studies should address in detail the injectability of the developed systems at physiological conditions, although it was demonstrated that by mixing the polymeric κ-carrageenan with the cross linker and subsequently adding the cell suspension, it was possible to extrude the system. In other words, κ-carrageenan hydrogels offer versatility in terms of administration given that it could be either applied as a "solid" construct with various specific shapes or delivered in an injectable way into a tissue defect. Simultaneously it is important to determine if tissue regeneration *in vivo* is achieved in a similar manner with or without *in vitro* pre-culturing of the cell laden hydrogels.

During all experimental trials it was observed a fast temporal weight loss of the 3D carrageenan hydrogel when exposed to environments rich in electrolytes, ions and cations that destabilize the hydrogel. This behavior, which is commonly observed in ionic hydrogels, can eventually be overcome through different strategies, namely by the addition of ions and/or repeating the cross-linking step in the presence of potassium chloride. Such improvement could allow a more precise control and tailoring of the degradation profile of the hydrogel structure. Other ways of preventing the dissolution of the hydrogels, is by taking advantage of the carrageenans chemical structure and modify it introducing groups which are more difficult to degrade. In fact, such strategy has been recently attained, by introduction of photocrosslinkable methacrylate moieties, enhancing the stability of these polysaccharides and thus obtaining systems with slower dissolution rates in physiological conditions. [1]

When compared to other hydrogels with similar characteristics and applications, namely alginate, agarose, gellan gum and ulvan, several distinct properties are observed. Carrageenan is extracted from red algae, like agarose, while alginate is obtained from a brown algae and ulvan from green algae. Gellan gum is a polysaccharide manufactured by microbial fermentation of the *Sphingomonas*

[1] Mihaila, S.M., et al., *Photocrosslinkable Kappa-Carrageenan Hydrogels for Tissue Engineering Applications.* Adv Healthcare Mater, 2012: p. n/a-n/a.

paucimobilis microorganism.[2] Carrageenan and alginate are ionic hydrogels, and its gelling mechanism is temperature dependent, like agarose, although in a different manner.

The registered values of the mechanical properties for κ-carrageenan based hydrogels are higher or within the range of the values found for other hydrogels used in similar cartilage regenerative approaches.[3] Moreover, compared to human articular cartilage which has a compressive modulus of 0.79 MPa, a shear modulus of 0.69 MPa, and a tensile modulus varying between 0.3 and 10.2 MPa, carrageenan hydrogels achieved up to 30–50% in strength and mechanical stiffness.[4]

Another advantageous feature of carrageenan hydrogel is that it enables to obtain a chondrocyte typical phenotype once cells are embedded in it, even without any chondrogenic stimulation agents. Such accomplishment was owed not only because of the hydrogels matrix, which is adequate to retain the rounded shape specific of chondrocytes, likely to enhance chondrogenesis, but can be equally due to the interaction between the sulphated groups of carrageenan and the cells. Thus, future studies should address in more detail the influence of the chemical composition of κ-carrageenan in chondrogenic differentiation. Moreover, polysaccharides like carrageenan are thought to protect growth factors from degradation and seem to interact with stem cells due to the carbohydrate glycochemistry, mediating cellular behavior.[5] The host reaction issue is a serious concern in current TE approaches and monocytes/macrophages are known to the typically present immediately after the implantation of a construct. Carrageenan is thought to affect the innate immune response in ways not yet defined, with the molecular weight and the sulphate composition playing important roles in such particular behavior.[6]

The inflammatory response is a key factor for tissue regeneration, thus targeting inflammation to support regeneration may be the solution. It is possible to sustain the concept that if understanding the signaling and activation pathways that regulate carrageenan inflammatory reaction may providing a rational for their use as therapeutic agents in treatment of inflammation-associated diseases.

In summary, the success of engineering carrageenan hydrogel for cartilage application was granted by the use of stem cells, namely human adipose tissue derived stem cells. It can be hypothesized that this behavior could be related to the undifferentiated stage of the cells, providing a better cellular outcome as compared to the human primary chondrocytes. Moreover, in terms of accessibility, scarcity,

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[2] Oliveira, J.T., et al., *Gellan gum: A new biomaterial for cartilage tissue engineering applications.* J Biomed Mater Res A, 2010. 93A(3): p. 852-863.

[3] C. Lee, et al., *Topics in tissue engineering*, in *The Influence of Mechanical Stimuli on Articular Cartilage Tissue Engineering*, N. Ashammakhi and R.L. Reis, Editors. 2006.

[4] Fisher, J.P., et al., *Thermoreversible hydrogel scaffolds for articular cartilage engineering.* J Biomed Mater Res A, 2004. 71(2): p. 268-74.

[5] Vollmers, H.P. and S. Brandlein, *Tumors: Too sweet to remember?* Mol Cancer, 2007. 6(1): p. 78.

[6] Bhattacharyya, S., et al., *Carrageenan-induced innate immune response is modified by enzymes that hydrolyze distinct galactosidic bonds.* J Nutr Biochem, 2010. 21(10): p. 906-913.

harvesting procedures and cell number available, stem cells particularly those obtained from the adipose tissue are outrunning the primary cells. It is well recognize that exists a spatial-temporal modeling, meaning that cells communicate differently with the surrounding releasing distinct biomolecules at different stage inducing variations in the cellular response. Thus, variables such as cell number, cell culture media formulation, the stage of differentiation and the biological properties of the hydrogels should be investigated in detail to optimize therapies based on the cell–laden hydrogels.

The option of using carrageenan natural hydrogels is relevant based on the gelling properties, the high chemical reactivity and the physical characteristics. The *in situ* gelation at physiological conditions is an important feature of carrageenan based hydrogels that may enable its application by injecting of the cell-laden systems directly into the tissue defect, in a minimally invasive manner. Such use of injectable hydrogels for cell delivery in cartilage regeneration therapies have attracted more and more attention, because of their promising advantages over pre-formed scaffolds. In any case, the chemical similarity to ECM may confer better biological performance and structural affinity, feature not yet fully exploited.

As final remark, the herein proposed and studied carrageenan hydrogel proved to be suitable and represent a valid alternative to the existing/standard hydrogels options. Overall, the research presented in this thesis has provided a general comprehension of the possible applications of carrageenan hydrogel and stem cells in cartilage engineering as well as a starting point and motivation for further research on the use of red algae based polymers.